The Molecular Mechanisms of Thrombus Growth and Stability

by

Hadil Owaynat

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Graduate Supervisory Committee:

Douglas Chandler, Chair
Norma Wilson-Rawls
Debra Baluch
Douglas Lake

ARIZONA STATE UNIVERSITY

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ABSTRACT

Thrombus (blood clot) formation is at the roots of hemostasis and pathological thrombosis. Although many studies have successfully elucidated the cellular and molecular mechanisms underlying thrombus formation, there is still a void in understanding the processes limiting thrombus growth beyond that needed for stabilization. As a hemostatic thrombus grows, its surface consisting primarily of platelets changes to that composed of fibrin, which mechanically stabilizes the thrombus. Formation of fibrin ceases after some time; however, it is unclear why this fibrin is non-thrombogenic. This is puzzling since fibrin is known to support strong integrin-mediated adhesion of both platelets and leukocytes *in vitro*. Therefore, it would be expected that the fibrin surface of hemostatic thrombi in the circulation also support accumulation of these cells and thus continuous thrombus growth or degradation. Nevertheless, many *in vivo* studies did not detect any accumulation of blood cells including platelets at the fibrin surfaces of thrombi. This finding suggests the existence of natural processes that modulate the adhesive properties of fibrin to ensure proper regulation of thrombus growth, stability and degradation. In this dissertation, I document and discuss the findings supporting the existence of anti-adhesive mechanisms and their physiological relevance in surface-mediated control of thrombus growth and stability. The studies discussed in my dissertation have the potential to establish a novel aspect of hemostasis. Furthermore, it may provide new insights into the intricate and dynamic interplay between the mechanisms underlying hemostatic balance, which is essential to understanding the dysfunction of this process during pathological conditions.

DEDICATION

I dedicate this dissertation to my family especially my parents for their love and support throughout my life and for always believing in me and being there.

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CHAPTER 1

INTRODUCTION

HEMOSTASIS AND THROMBOSIS

The process by which blood loss, a result of vascular damage or injury, is stopped and normal blood flow is restored is termed hemostasis. Hemostasis relies on a number of tightly controlled and complex interactions between circulating platelets, the vessel wall, coagulation factors, natural anticoagulants, and fibrinolytic proteins. This physiological response is a multistep dynamic process that involves three main stages: primary hemostasis, secondary hemostasis, and fibrinolysis (clot dissolution). These three stages involve a series of delicately balanced physical and biochemical changes. Primary hemostasis occurs when blood vessels undergo vasoconstriction and circulating platelets activate, aggregate (clump together), and accumulate at the site of vascular injury. Platelet aggregation results in the formation of a platelet plug that acts as a temporary seal. This initial response is followed by a cascade of biochemical (enzymatic and non-enzymatic) reactions, referred to as the coagulation cascade, in which a series of inactive soluble zymogens and coagulation factors become active, eventually leading to the production of thrombin which mediates the generation of an insoluble cross-linked fibrin network that stabilizes and strengthens the platelet plug (secondary hemostasis). The coagulation cascade is initiated by activation of one of two pathways, the extrinsic and intrinsic pathway. The extrinsic pathway is triggered in response to tissue injury and exposure of highly expressed tissue factor (TF) on the surface of sub-endothelial cells. Alternatively, the intrinsic pathway is triggered by exposure of sub-endothelial collagen

and platelet-derived polyphosphates. Ultimately, the two pathways converge to form a common pathway that results in thrombin generation and fibrin formation (Colman, 2006;Versteeg, Heemskerk, Levi, & Reitsma, 2013).

The platelet plug together with the deposited fibrin form a thrombus or a blood clot; the entire process is referred to blood coagulation (i.e. changing of blood from a fluid to a solid state). In its basal state, blood remains in liquid form, despite the presence of a vast excess of coagulation proteins and circulating platelets. This is due to a number of adaptive measures that suppress the coagulation machinery. Normally, healthy endothelium is devoid of any pro-thrombogenic components such as TF or collagen, both platelets and coagulation proteins circulate in an inactive state, and the fast blood flow and shear rate ensures removal of any activated proteins and/or platelet aggregates. (Colman, 2006; Versteeg, Heemskerk, Levi, & Reitsma, 2013; Thon & Italiano, 2010; Hartwig & Italiano, 2003).

Finally, following tissue repair, components of the fibrinolytic system begin degrading fibrin and dissolving the thrombus (i.e. changing of blood from a solid to a fluid state) in order to limit the clot size and restore local blood flow. Altogether, these highly coordinated hemostatic interactions ensure the formation of a thrombus that is structurally strong and stable enough to seal the breach, stop the bleeding, and restore local hemodynamics but not overly robust to cause vessel occlusion. An overview of these processes is shown in Fig. 1.

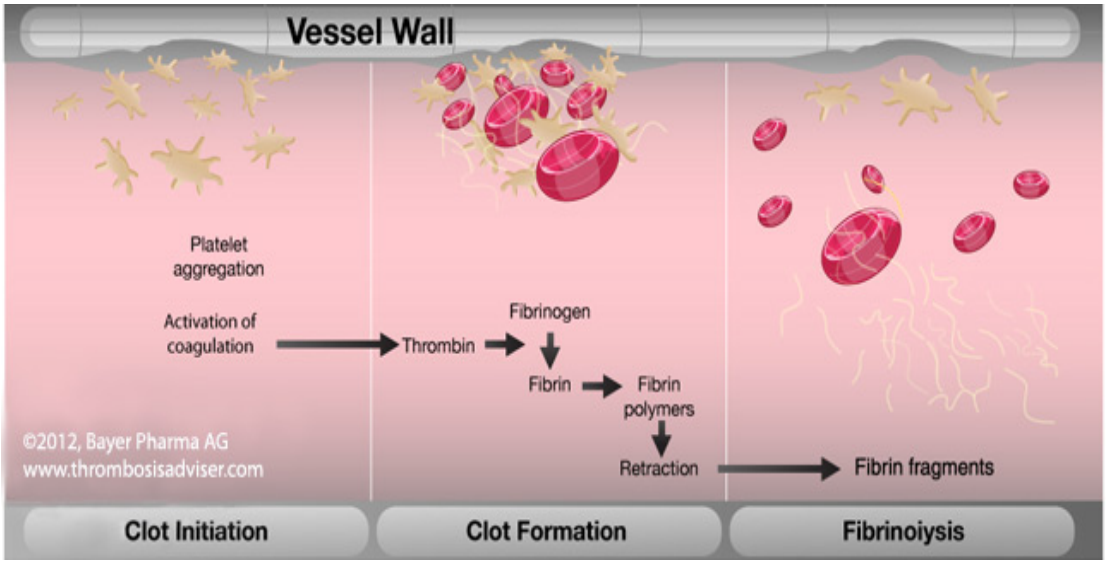


Figure 1. The hemostatic process. Hemostasis depends on an intricate interplay of cellular and enzymatic activities that mediate the formation of a stable thrombus at the site of vascular injury. After tissue repair, the thrombus undergoes degradation and dissolution by a process known as fibrinolysis. (www.thrombosisadvisor.com)

This highly regulated, localized, and complex process, designed to preserve the integrity of the circulation, involves an intricate balance between the pro-thrombogenic (pro-coagulant) events which serve to enlarge and strengthen the thrombus and anti-thrombogenic (anti-coagulant) events which limit the growth of the thrombus and ultimately lead to its dissolution. However, when pathologic processes overwhelm the regulatory mechanisms of hemostasis, disturbances occur. Disturbances due to alterations in cellular and/or protein composition at any step along the hemostatic pathway may result in either bleeding or thrombosis. Although hemostasis and thrombosis trigger similar cellular and molecular mechanisms, thrombotic events often lead to uncontrollable thrombus growth resulting in vascular occlusion and obstruction of blood flow. A major concern of thrombosis is when the occlusive thrombus breaks free from

the site of injury and travels along the vessel where it becomes lodged somewhere else. This is known as a thromboembolism. Hemostatic dysfunction is often observed in cardiovascular disease (CVD). CVD is the leading cause of morbidity and mortality in the United States, accounting for an average of one death every 40 seconds, and is a growing problem in many countries worldwide. Abnormal thrombi can produce severe pathologies such deep venous thrombosis (DVT), pulmonary embolism (PE), and coronary artery thrombosis leading to myocardial infarction and stroke. Therefore, elucidation of the molecular and cellular mechanisms that mediate hemostatic balance is crucial to understanding the dysfunction of this process under pathological conditions.

PLATELETS AND THROMBUS FORMATION

A thrombus is mainly composed of a core of tightly packed platelet aggregates surrounded by a fibrin network. Platelets are discoid shaped, anucleate cytoplasmic fragments, 2-3 μm in diameter (see Fig.2), released into the circulation by megakaryocyte precursor cells residing in the bone marrow. The physiological platelet count is $150 – 400 \times 10^3$ per microliter of blood with a platelet life span of 7 to 10 days. Despite their lack of a nucleus and their small size, platelets are remarkably versatile. Platelets have two elaborate channel systems, the open canalicular system (OCS) and the dense tubular system (DTS). The OCS is a surface-connected channel system that serves as a passageway between the platelet cytosol and the plasma, allowing internalization of substances from the plasma into platelets and externalization of platelet secretory products. DTS provides a site for calcium sequestration and localization of important

metabolic enzymes. Furthermore, their plasma membrane contains a variety of surface receptors that are fundamental to the platelet hemostatic function. The most well studied platelet surface receptors fall into three categories: integrins, glycoproteins, and G-protein coupled receptors (GPCRs). An important characteristic of integrins is their ability to bi-directionally transmit signals across the platelet plasma membrane, which acts as dynamic link between the platelet's intracellular and the extracellular environment. These receptors not only provide a localized reactive surface that facilitates the assembly and activation of coagulation factors but also sense activating agonists that mediate platelet signaling, adhesion, and aggregation. Platelets also contain two major storage granules, alpha and dense granules, whose contents are essential for normal platelet activity. When activated, platelets release the contents of their granules at sites of vascular injury to induce controlled thrombus formation and growth thus initiating vascular repair and restoring hemostatic balance. The alpha granules contain a number of adhesion molecules such as fibrinogen, fibronectin, von Willebrand factor (vWF), P-selectin, integrin $\alpha_{IIb}\beta_3$, integrin $\alpha_V\beta_3$, as well as a number of coagulation factors and cofactors (e.g. V, XI, and XIII), chemokines, and inhibitory proteases. The dense granules contain small molecules, such as adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin and calcium, which act as prothrombotic mediators important for the activation, aggregation and recruitment of additional platelets to the site of injury. Platelets also have lysosomes, which contain hydrolases that may play a role in eliminating aggregates of platelets from the circulation. (Thon & Italiano, 2010; Hartwig & Italiano, 2003).

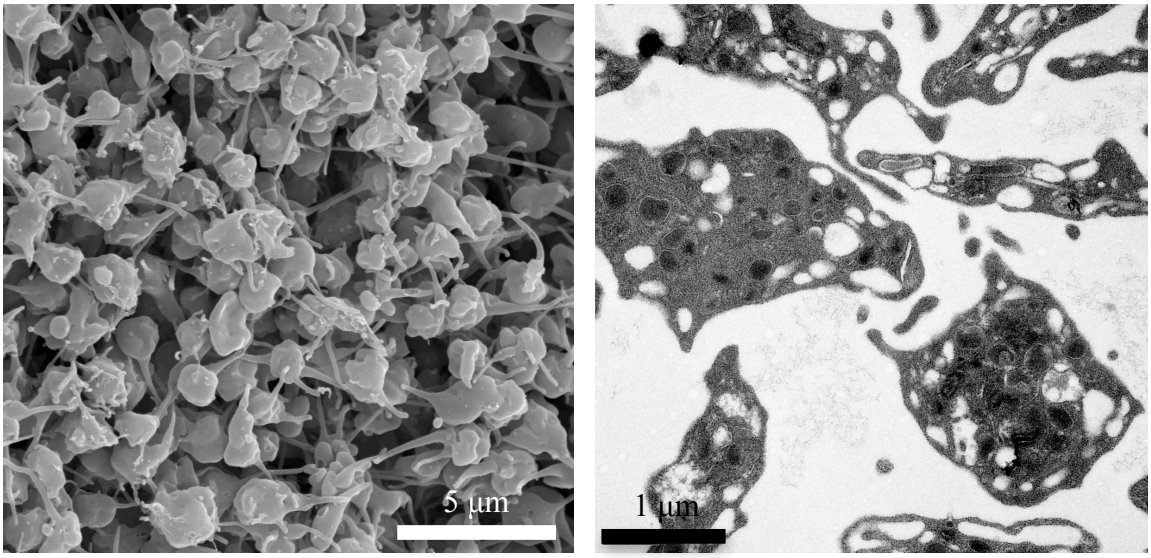


Figure 2. Scanning and transmission electron micrographs of platelets. The left image is a scanning electron micrograph (SEM) of a platelet aggregate. Platelets interact with one another via long thin pseudopods. The right image is a transmission electron micrograph (TEM) of platelets seen in cross section. The cytoplasm contains channels and granules of different sizes.

It is well known that platelets play a pivotal role in the maintenance of hemostasis. However, platelets are also instrumental to the excessive thrombosis and vessel occlusion that underlies the pathogenesis of stroke, acute coronary syndrome, and peripheral arterial disease (Gosavi & Mukherjee, 2013; Fitzgerald, Roy, Catella, & FitzGerald, 1986; Epstein, Fuster, Badimon, Badimon, & Chesebro, 1992; del Zoppo, 2000). Normally platelets remain quiescent, or in an inactive state, due to the lack of activating signals as wells as endothelial cell signaling and production of antithrombotic agents such as nitric oxide and prostacyclins (Moncada & Higgs, 2006). However, upon vascular injury and endothelial damage, endothelial cells

increase the release and expression of pro-coagulant and adhesive molecules such as tissue factor (TF) and vWF that promote the recruitment and activation of circulating platelets that results in formation of a platelet plug at the site of injury. Formation of the plug is accompanied by a positive feedback cycle of platelet activation and agonist-induced aggregation of additional platelets resulting in further platelet activation.

Leukocytes may influence platelet activity via the release of pro-coagulant and anti-coagulant molecules. Moreover, leukocytes are recruited to the growing thrombus via interaction of the leukocyte PSGL-1 with P-selectin, which is expressed by activated platelets. These leukocytes are thought to play a role in promoting fibrin deposition (Palabrica et al., 1992). Red blood cells (RBCs) have been also shown to contribute to the hemostatic function of platelets during injury. At high shear rate, RBCs increase the frequency of platelet-platelet and platelet-endothelial cell interactions by promoting flow of platelets towards the vessel wall. This is referred to as platelet margination. RBCs also release some amounts of ADP, thereby facilitating the ADP-induced platelet activation and aggregation (Crowl & Fogelson, 2010; Reasor Jr, Mehrabadi, Ku, & Aidun, 2013). Therefore, blood cells and the vasculature play a cooperative role in facilitating the hemostatic function of platelets.

Exposure of extracellular matrix (ECM) proteins such as laminin, fibronectin, and collagen type I at the site of vascular wall injury provide a surface that not only activates platelet receptors but also serves as a surface to which platelets must adhere (Versteeg, Heemskerk, Levi, & Reitsma, 2013; Z. Ruggeri, 1997; Z. M. Ruggeri & Mendolicchio, 2007; Bergmeier & Hynes, 2012). Platelet adhesion is dependent on the synergistic function of multiple platelet receptors and agonists. As shown in Figure 3, the binding

and immobilization of vWF, via vWF-specific binding sites, on the exposed collagen creates a surface with which platelets can loosely interact. Under high shear rate, vWF undergoes a shear-induced conformational change that enhances its binding to platelets (Singh, Themistou, Porcar, & Neelamegham, 2009). vWF coating the site acts as a solid ligand for glycoprotein Ib (GPIb) that is part of the GPIb/IX/V complex on the platelet surface (Canobbio, Balduini, & Torti, 2004). This labile interaction decelerates platelets from the rate of blood flow causing them to roll across the sub-endothelium to a stop and get captured. After the initial tethering, platelets rapidly adhere to the sub-endothelial collagen through their integrin receptor $\alpha_2\beta_1$. Platelet adhesion to collagen is further mediated by an additional collagen receptor, the immunoglobulin-like receptor glycoprotein VI (GPVI), which also plays an important role in inducing platelet activation and aggregation (Siljander et al., 2001). Laminin and fibronectin are also known to support platelet adhesion and spreading via interaction with their integrin receptors $\alpha_6\beta_1$ and $\alpha_5\beta_1$, respectively (Versteeg, Heemskerk, Levi, & Reitsma, 2013).

Following the initial adhesion, platelets undergo a range of biochemical and morphological responses. Platelet surface integrins undergo a conformational change, setting the ligand-binding site into an active high affinity/avidity state, due to cytoplasmic signaling and phosphorylation of their cytoplasmic domains. The activated integrins mediate both outside-in and inside-out signaling which allow for multiple adhesive receptor-ligand interactions. Platelets also undergo a physical change in shape, from discoid to pseudopodium formation, through extensive cytoskeletal rearrangements that result in platelet spreading and flattening. This process is initiated by a rise in cytosolic calcium due to mobilization of intracellular calcium stores and/or

extracellular calcium influx. The sustained rise in intracellular calcium also influences the distribution of plasma membrane phospholipids and their exposure at the outer membrane surface. Platelet spreading allows closer and firmer adhesion and the formation of a platelet monolayer that covers the exposed sub-endothelium. These processes require precise coordination of several intracellular signaling pathways to ensure synchronization of the activation state of platelet and thus adhesiveness of their receptors/integrins (Versteeg, Heemskerk, Levi, & Reitsma, 2013; Jackson, Nesbitt, & Kulkarni, 2003; Gibbins, 2004; Stegner & Nieswandt, 2011; Savage & Ruggeri, 2007)

Activated platelets degranulate releasing an array of adhesive ECM proteins as well as soluble agonists such as ADP, thromboxane A2 (TxA2), and epinephrine, which act on G-protein coupled receptors, $P2Y_{12}$/$P2Y_1$, TP, and α_2A, respectively. Platelets also release serotonin, a vasoconstrictor that limits blood loss from the site of injury. Local accumulation of agonists, which act in an autocrine/paracrine fashion, both increases the activation level of the firmly adhered platelets and promotes the activation and recruitment of additional platelets (Versteeg, Heemskerk, Levi, & Reitsma, 2013; Nesbitt et al., 2009). Platelet accumulation at the site of injury occurs in a spatially and temporally restricted pattern resulting in the formation of a core of tightly packed platelets surrounded by heterogeneous layers of less-packed platelets (I. Munnix, Cosemans, Auger, & Heemskerk, 2009; Stalker et al., 2013). ADP plays an important role in initiating and maintaining activation of integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa, CD41/CD61) that is necessary for the formation of stable and tight platelet-platelet contacts. Platelets interact with one another via cross-linking of their activated $\alpha_{IIb}\beta_3$ integrins with the plasma protein fibrinogen to form the platelet aggregates that make up

the platelet plug. $\alpha_{IIb}\beta_3$ integrin also supports platelet interactions with a variety of adhesive glycoproteins (GPs) other than fibrinogen, including vWF, thrombospondin, fibronectin, and vitronectin (Versteeg, Heemskerk, Levi, & Reitsma, 2013). This process of bridging of adjacent platelets provides a protective environment for the accumulation of soluble agonists that are important for the formation of a stable platelet plug that will contribute to endothelial healing and wound repair (Brass, Wannemacher, Ma, & Stalker, 2011). Furthermore, formation of the platelet plug is spatially coordinated with the activation of the blood coagulation system.

Platelet activation, subsequent intracellular signaling and the increase of intracellular calcium levels induces exposure of plasma membrane phospholipids, specifically phosphatidylserine (PS), at the outer leaflet of the membrane thus creating a negatively charged platelet surface. This surface links the processes of platelet activation and thrombin generation. It provides a pro-thrombotic (procoagulant) surface that favors the assembly and association of a series of activated coagulation factors into enzyme complexes known as the tenase and pro-thombinase complex. This surface-mediated assembly of the enzyme complexes protects the activated factors from local inhibitors. The complex serves to catalyze the conversion of the zymogen prothrombin into its active form thrombin (Versteeg, Heemskerk, Levi, & Reitsma, 2013). Local production of thrombin onto the surface of platelet aggregates is necessary to maintain a positive feedback loop that reinforces the activation of adherent platelets and the recruitment of additional platelets to the growing platelet plug, further stimulating the coagulatory process. Thrombin acts as a potent platelet activator via its proteolytic action on the protease-activated receptors (PARs), PAR-1 and PAR-4, found on the surface of platelets

(Versteeg, Heemskerk, Levi, & Reitsma, 2013; Coughlin, Vu, Hung, & Wheaton, 1992). The interaction of thrombin with its PAR1 and PAR4 receptors results in modulation of ligand affinity of the $\alpha_{IIb}\beta_3$ integrin, thus promoting further $\alpha_{IIb}\beta_3$ activation and platelet aggregation. Moreover, the interaction of thrombin with plasma fibrinogen eventually results in the formation of insoluble fibrin that is deposited on the surface of platelet aggregates in a form of a network (Bruce Furie & Furie, 2005; Shahrokh Falati, Peter Gross, Glenn Merrill-Skoloff, Barbara C Furie, & Bruce Furie, 2002).

Sine the extent of the thrombus must remain confined to the immediate surrounding area of the vascular breach, thrombin generation is controlled and regulated by three important mechanisms including anti-thrombin III (AT III), tissue factor pathway inhibitor (TFPI), and activated protein C (APC). The level of thrombin within the growing thrombus regulates not only the fibrin structure but also the compactness of the platelet plug. Thrombin also mediates the platelet-dependent clot retraction, which is necessary for compacting the platelet plug and thus the entire thrombus on itself (Brass, Zhu, & Stalker, 2005). This functions to concentrate the thrombus to the site of injury thereby closing the breach and clearing the obstructed vessel for renewed blood flow. Fibrin assembly together with the tight platelet-platelet contacts restricts the diffusion of plasma factors, thus preventing premature fibrinolysis of the growing thrombus.

The fibrin network endows the platelet plug with mechanical stability and arrests bleeding. If the resultant thrombus is unstable then it can be prematurely detached or embolized. Alternatively, if the thrombus continues to be thrombogenic it can become occlusive. Furthermore, variations or disturbances in platelet reactivity are associated with various pathologies such as atherosclerosis, thrombotic cardiovascular disorders,

and inflammation leading to morbidity and mortality. Thus, appropriate regulation of platelet function is of paramount importance because it maintains the balance between hemostasis and thrombosis (I. C. Munnix et al., 2007).

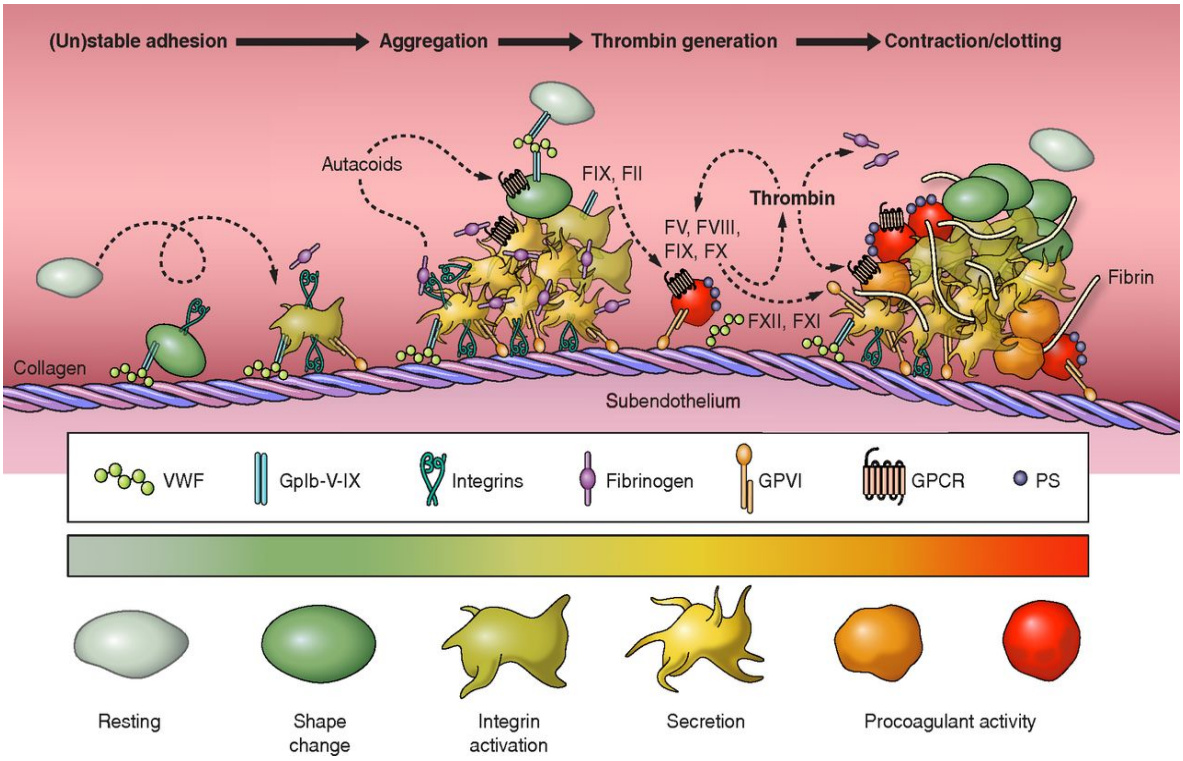


Figure 3. Stages of platelet activation and thrombus formation. Formation of a platelet plug (i.e. platelet aggregate) at the site of injury is a result of complex platelet-platelet interactions mediated by platelet receptors, plasma and extracellular matrix-derived adhesive proteins, as well as platelet-derived agonists and adhesive proteins. Concurrent with platelet activation and aggregation is the activation of the coagulation cascade. The sequential activation of a series of biochemical reactions results in the production of thrombin, the serine protease responsible for converting soluble fibrinogen into an insoluble fibrin. Fibrin is deposited around the platelet plug as a cross-linked network resulting in the formation of a stable thrombus. Figure source: (Versteeg, Heemskerk, Levi, & Reitsma, 2013).

FIBRINOGEN, FIBRIN, AND FIBRINOLYSIS

Plasma fibrinogen is a large glycoprotein (340 kDa) synthesized in the liver and circulates at a concentration of 2.5-3 mg/ml. In a healthy individual, the half-life of fibrinogen is 3 to 4 days. Fibrinogen is a disulfide-linked dimer composed of three pairs of non-identical polypeptide chains Aα, Bβ, and γ (see Fig. 4). The two subunits Aα and Bβ are aligned in an antiparallel manner forming a trinodular arrangement of the six chains. The central region (i.e. the E region) is formed by the N-termini of all six chains held together by disulfide bonds. This region contains four thrombin-sensitive sites (Yermolenko, Lishko, Ugarova, & Magonov, 2010).

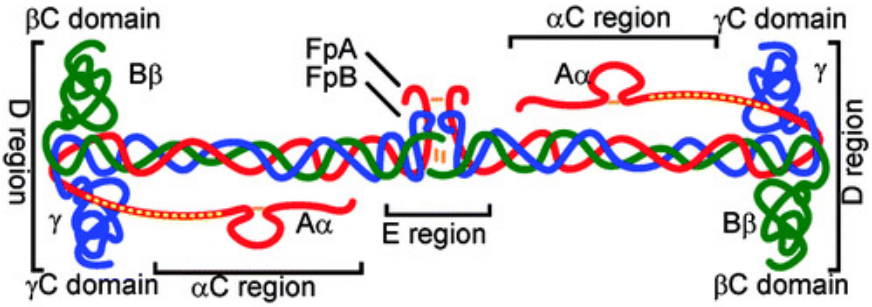


Figure 4. Schematic diagram of fibrinogen structure. The three chains, Aα, Bβ, and γ in the fibrinogen molecule are shown in red, green, and blue, respectively. FpA and FpB are small fibrinopeptides in the central E region, which are cleaved by thrombin to initiate fibrin polymerization. The peripheral D regions are composed of the βC and γC domains. Figure source: (Yermolenko, Lishko, Ugarova, & Magonov, 2010).

Thrombin is a 37 kDa serine protease generated by the proteolytic cleavage of the zymogen prothrombin, an essential step of the coagulation cascade. Thrombin mediates the removal of the two fibrinopeptides A and B from the N-terminal ends of the Aα and Bβ chains, thus exposing the 'knobs' on the E domain which can interact with the 'holes' always present on the D domains. More specifically, cleavage of fibrinogen by thrombin at Arg16-Gly17 in the N-termini of the Aα -chains releases two fibrinopeptides A (FpA) thereby exposing the polymerization sites (residues 17-20). These sites bind to the complimentary sites available in the D region of fibrinogen to form double-stranded protofibrils. Subsequent thrombin cleavage of two other fibrinopeptides B (FpB) at Arg14-Gly15 from the Bβ chain exposes additional polymerization sites. Spontaneous polymerization (i.e. formation of multiple 'hole-knob' interactions) of the fibrin monomers formed results in the assembly of a half staggered arrangement of fibrin fibrils that grow longitudinally and laterally with branch points forming and interconnecting the fibers. The fibers are covalently cross-linked by the thrombin activated factor XIIIa (FXIIIa) leading to the formation of a three-dimensional viscoelastic network (see Fig. 4). Factor XIIIa crosslinks the glutamine residues on one fibrin molecule to the lysine residues on another fibrin molecule by forming strong isopeptide bonds that strengthens the thrombus making it resistant to chemical or physical damage. The cross-linked fibrin forms a mesh atop of the platelet plug thereby completing the clot. This fibrin network endows the clot with mechanical stability and arrests bleeding (M. Mosesson, 2005; John W Weisel & Rustem I Litvinov, 2013). A schematic representation of the steps involved in fibrin formation is shown in Fig.5.

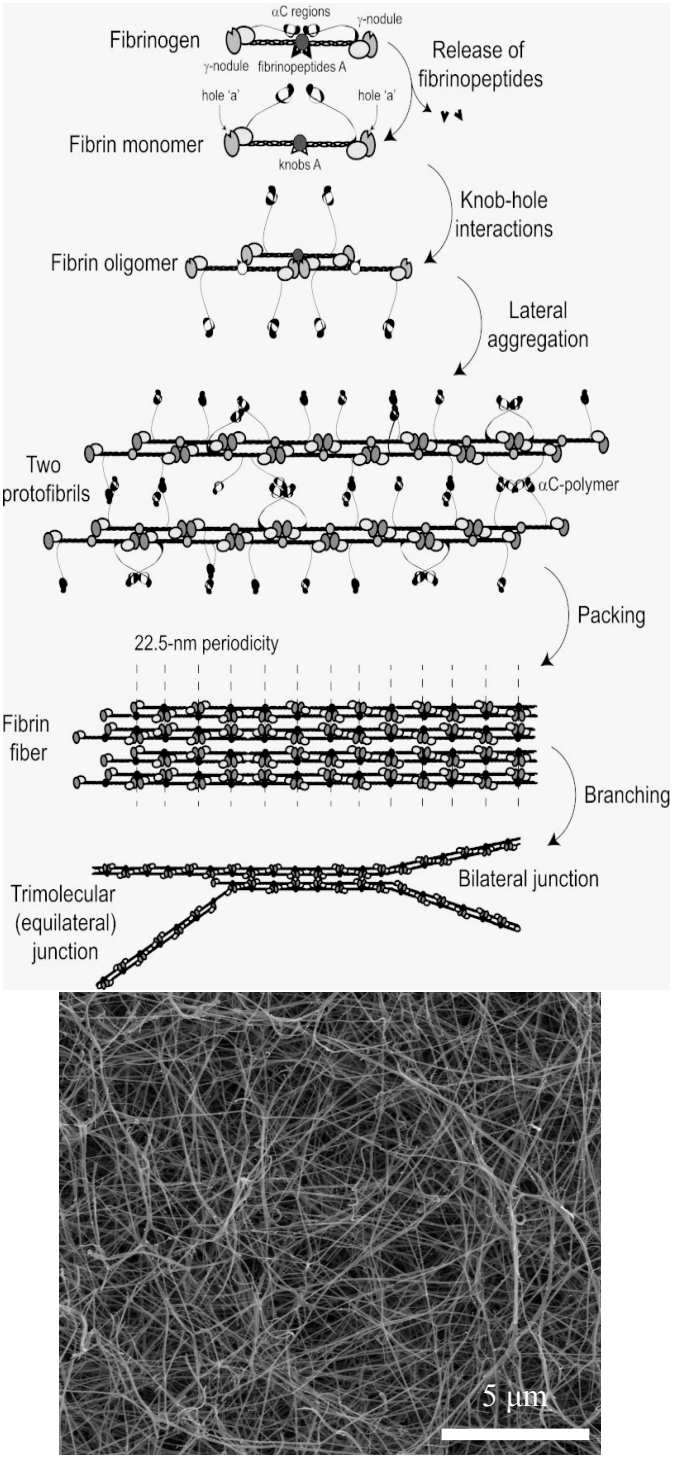


Figure 5. Schematic diagram of fibrin formation. The thrombin-mediated cleavage of fibrinopeptides A and B results in the generation of fibrin monomers. The spontaneous polymerization of fibrin monomers eventually leads to the formation of an insoluble cross-linked network of fibrin. Figure source: (John W Weisel & Rustem I Litvinov, 2013). The image below is a SEM of a fibrin network.

Fibrin deposition continues until it reaches a plateau and the entire thrombus is stabilized. The concentrations of both thrombin and fibrinogen present during thrombus formation influence the structure, density, and stability of the fibrin network. For example, elevated thrombin or fibrinogen levels results in firmer fibrin clots, which are more resistant to fibrinolysis and are associated with an increased risk of arterial or venous thrombosis-related disease. In contrast, impaired thrombin or fibrinogen generation results in fibrin clots that are less stable and more susceptible to fibrinolysis and spontaneous bleeding (Blombäck, Carlsson, Fatah, Hessel, & Procyk, 1994; Wolberg, Monroe, Roberts, & Hoffman, 2003; van Hylckama Vlieg & Rosendaal, 2003; Wolberg, 2007; Undas & Ariëns, 2011; Ariëns, 2013).

Numerous studies have identified an array of binding sites on fibrinogen/fibrin necessary for interaction with a variety of soluble plasma proteins essential in thrombus formation, stabilization, and fibrinolysis. These binding proteins include thrombin, fibronectin, factor XIII (FXIII), tissue-type plasminogen activator (t-PA), plasminogen, and plasmin (Makogonenko, Tsurupa, Ingham, & Medved, 2002; Meh, Siebenlist, Brennan, Holyst, & Mosesson, 2001; Siebenlist, Meh, & Mosesson, 1996; Lucas, Fretto, & McKee, 1983). Fibrinogen/fibrin also contains recognition sequences for cellular integrins including $\alpha_{IIb}\beta_3$, $\alpha_M\beta_2$, and $\alpha_V\beta_3$, which mediate fibrinogen/fibrin interactions with platelets, leukocytes, and endothelial cells, respectively. These interactions may regulate platelet activation and aggregation, leukocyte function, as well as mediate thrombus anchorage to the endothelium (Altieri, Bader, Mannucci, & Edgington, 1988; Flick et al., 2004; Yakovlev & Medved, 2009; M. Mosesson, 2005).

Following tissue repair, however, the thrombus must be disassembled and removed in order to limit intravascular thrombotic processes and restore normal blood circulation. The process of fibrin degradation and thrombus dissolution is known as fibrinolysis. The fibrinolytic system is crucial for the lysis of thrombi as it plays an important role in restricting thrombus growth beyond the site of injury by acting as a counter-regulatory mechanism to the coagulation cascade. The fibrinolytic system is composed of several serine proteases whose activity is carefully regulated by a balance of activators and inhibitors (see Fig. 6). The main enzyme responsible for fibrin degradation and solubilization is plasmin, which is produced by the activation of its zymogen plasminogen. The plasmin-mediated degradation exposes several C-terminal lysine sites on the fibrin surface that allows binding of additional plasminogen and its activators, thus increasing the rate of lysis. Plasminogen, a 92 kDa glycoprotein, is synthesized in the liver and circulates in plasma at a concentration of 100-160 μg/mL. Plasminogen activation and the generation of plasmin is catalyzed by two physiological plasminogen activators: tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA). Both t-PA and u-PA convert plasminogen into plasmin by cleaving a single Arg561–Val562 peptide bond. t-PA mediates the activation of fibrin-bound plasminogen involved in fibrin dissolution in the circulation. In contrast, u-PA mediates the activation of cell-bound plasminogen via its binding to a specific cellular receptor, u-PAR (Chandler, 1996; Lijnen, 2001).

The rate of fibrinolysis is influenced by a number of factors including rate of blood flow, local levels of activators and inhibitors, the amount of platelets included within the thrombus, the rate of fibrin formation, and the architecture of the fibrin

network. To keep the fibrinolytic system from interfering with the vital processes involved in the formation and stability of a hemostatic thrombus, it must be constrained by regulatory mechanisms. Down-regulation of the fibrinolytic system occurs either at the level of plasminogen activators by specific plasminogen activator inhibitor (PAI) such as PAI-1, or at the level of plasmin by α2-antiplasmin. α2-antiplasmin can bind to fibrin, via the action of FXIIIa, thus facilitating local inhibition of fibrinolysis. The fibrinolytic system, like blood coagulation, is under tight regulation to ensure hemostatic balance. Any dysregulation of this system may result in an increased or impaired fibrinolytic function leading to the propensity of bleeding or thrombosis. Indeed, numerous *in vivo* studies have shown that deficiencies or absences of PAI-1 contribute to accelerated fibrinolysis and bleeding. In contrast, high levels of PAI-1 are associated with spontaneous thrombus formation and increased risk of thrombotic disease (Thelwell & Longstaff, 2007; Prins & Hirsh, 1991).

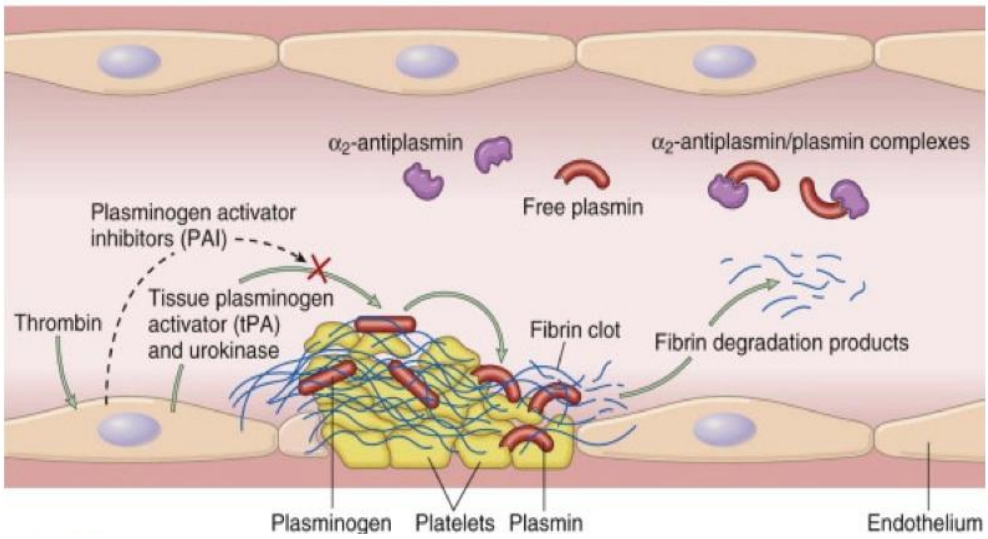


Figure 6. Schematic diagram of the fibrinolytic system. The fibrinolytic process depends on the enzymatic activity of a group of serine proteases. Plasmin degrades fibrin eventually solubilizing the entire thrombus. Fibrinolytic balance is maintained by anti-fibrinolytic enzymes which act either at the level of plasminogen activators (PAI) or at the level of plasmin (α2-antiplasmin). Figure source: (Mitchell & Kumar, 2012).

Focus of this Dissertation: Regulation of Thrombus Growth and Stability

Although many of the thrombogenic and anti-thrombogenic mechanisms regulating the hemostatic process have been successfully identified and extensively studied, there still a void in knowledge and understanding of how the hemostatic process works, more specifically how the growth of a thrombus is terminated and what cellular and molecular mechanisms are involved in regulating thrombus growth and stability.

Under physiological conditions, it has been observed that the surface of a hemostatic thrombus, mainly composed of fibrin, doesn't support the accumulation and adhesion of blood cells including platelets. This observation is puzzling as it contradicts the *in vitro* findings showing that fibrin is a highly adhesive substrate for platelets as well as for leukocytes. According to these *in vitro* studies, it would be expected that the surfaces of stabilized hemostatic thrombi in the circulation do support accumulation of these cells and thus continuous thrombus growth or degradation. However, this is not the case. Rather, it seems that the thrombus surface has non-adhesive properties, which do not allow further accumulation of circulating blood cells. This suggests the existence of natural processes that act on the surface of hemostatic thrombi making it non-adhesive, thus ensuring proper regulation of thrombus growth, stability and dissolution. By restricting platelet accumulation on the surface of fully formed thrombi, uncontrollable growth and pathological thrombosis can be avoided. Likewise, regulation of leukocyte (known to contain fibrinolytic enzymes) accumulation protects thrombi from premature fibrinolysis. The focus of this dissertation is to document and discuss findings that support the existence of anti-adhesive mechanisms operating on the surface of stabilized hemostatic thrombi, and their physiological relevance in regulating thrombus growth,

stability and timely dissolution. This surface-mediated control of thrombus growth and stability may represent a new aspect of hemostasis, which would set the direction for future research. Collectively, this dissertation provides new insights into the intricate interplay between the mechanisms underlying hemostatic balance, which is essential to understanding the pathological conditions contributing to cardiovascular disease.

CHAPTER 2

FIBRINOGEN NEUTRALIZES THE ANTI-ADHESIVE EFFECT OF FIBRIN SURFACE-BOUND PLASMINOGEN

ABSTRACT

The adhesion of platelets and leukocytes to the surface of fibrin clots is significantly reduced in the presence of fibrinogen and plasminogen, highlighting their potential role in surface-mediated control of thrombus growth and stability. Adsorption of fibrinogen onto fibrin clots renders their surfaces non-adhesive, while the activation and conversion of fibrin surface-bound plasminogen into plasmin results in degradation of a superficial fibrin layer leading to cell detachment. Even though the mechanisms by which these proteins exert their anti-adhesive effects are different, the result is the same. They make surfaces mechanically unstable and incapable of supporting firm cell attachment. Since fibrin clots in the circulation are exposed to both plasma proteins, their combined effect on cell adhesion was studied. Artificial fibrin clots were coated with plasminogen and its activation by transiently adherent U937 monocytic cells, in the presence of increasing concentrations of fibrinogen. Plasminogen activation was detected either by ^{125}I-labeled fibrin degradation products or plasmin amidolytic activity. Unexpectedly, the combined anti-adhesive effects were not additive. Fibrinogen strongly reduced the anti-adhesive effect of plasminogen. Our investigations revealed that fibrinogen masked the U937 cell-mediated activation of fibrin-bound plasminogen. Confocal microscopy showed that fibrinogen accumulates in the thin superficial layer of a clot, where it exerts its blocking effect on plasminogen activation. The results point to an intricate and dynamic interplay

between the fibrinogen- and plasminogen-dependent anti-adhesive processes, which may contribute to the mechanisms that control surface thrombogenicity of hemostatic thrombi.

INTRODUCTION

Upon vascular injury, thrombus formation, an intricately regulated dynamic process, is initiated by the adhesion of platelets to the exposed sub-endothelial matrix. This is followed by a series of signaling events that induce platelet activation and further platelet accumulation and aggregation. The formation of the platelet plug is then followed by thrombin-mediated generation of a fibrin network that structurally stabilizes the entire thrombus. Although the accumulation of platelets ceases shortly after the injury, fibrin deposition on and around the platelet plug continues for several hours, resulting in the formation of an extensive three-dimensional fibrin network (Hattori, Watanabe, & Izumi, 1978; Baumgartner, 1973; van Ryn, Lorenz, Merk, Buchanan, & Eisert, 2003; Beygui, Collet, Nagaswami, Weisel, & Montalescot, 2006). The deposition of fibrin on the surface of experimental hemostatic thrombi has been recently documented using *in vivo* imaging (S. Falati, P. Gross, G. Merrill-Skoloff, B. C. Furie, & B. Furie, 2002; Kamocka et al., 2010; Cooley, 2011). Furthermore, it has also been shown that the surface of such fibrin-coated thrombi contains very few adherent platelets and leukocytes (van Ryn, Lorenz, Merk, Buchanan, & Eisert, 2003; Groves et al., 1982; van Aken & Emeis, 1982; McGuinness et al., 2001). The latter finding contradicts numerous *in vitro* findings showing that fibrin is a highly adhesive surface for these blood cells (Wright et al., 1988; Kuijper, Gallardo Torres, van der Linden, et al., 1997; Kuijper, Gallardo Torres, Lammers, et al., 1997). However, it seems intuitive that fibrin deposited on the surface of

stabilized hemostatic thrombi must be non-adhesive for blood cells. This is because uncontrollable platelet adhesion, accumulation, and their subsequent activation would result in continuous thrombus growth and potentially vessel occlusion. Likewise, uncontrollable adhesion of leukocytes, which are known to contain potent fibrinolytic enzymes, could lead to premature thrombus lysis and destabilization resulting in bleeding. Therefore, cell adhesion must be tightly regulated to allow early hemostasis to proceed unchallenged until optimal thrombus growth and stability is established. This suggests that a proper balance between adhesive and anti-adhesive mechanisms operating on the fibrin surface of thrombi in circulation is necessary to ensure control of thrombus formation, stability and timely dissolution. Despite the clarity of this idea and the supporting experimental evidence, the finding that the fibrin surface in circulation is non-adhesive for blood cells remains poorly appreciated.

Recently, we have identified two natural processes that may contribute to the anti-adhesive mechanisms operating on the surface of fibrin clots (Lishko, Burke, & Ugarova, 2007; Lishko, Yermolenko, & Ugarova, 2010). The first anti-adhesive process depends on the formation of a nanoscale multilayered fibrinogen matrix that is characterized by weak adhesion forces and increased extensibility (I. S. Yermolenko et al., 2010). Origin of the non-adhesive properties of fibrinogen matrices lies in its inability to transduce strong integrin-mediated mechanical forces, which results in insufficient intracellular signaling and weak cell spreading (Lishko, Yermolenko, & Ugarova, 2010; I. S. Yermolenko et al., 2010). Indeed, it was observed that adsorption of fibrinogen on various surfaces including fibrin clots dramatically reduces adhesion of platelets and leukocytes (Kuijper, Gallardo Torres, Lammers, et al., 1997; Lishko, Burke, & Ugarova,

2007; Lishko, Yermolenko, & Ugarova, 2010; Endenburg, Lindeboom-Blokzijl, Zwaginga, Sixma, & de Groot, 1996). The second anti-adhesive process depends on the activation of plasminogen, which also accumulates in a thin layer on the fibrin surface, by components of the plasminogen activation system assembled on transiently adherent blood cells, including monocytes and platelets (Lishko et al., 2010). Consequently, the generated plasmin degrades and solubilizes the fibrin surface resulting in cell detachment under flow. Although the anti-adhesive effects of fibrinogen and plasminogen involve non-proteolytic and proteolytic means, respectively, they are based on a common underlying mechanism, which is modification of the fibrin clot surface and creating a mechanically unstable substrate that does not support firm integrin-mediated cell adhesion and spreading.

Since fibrin on the surface of thrombi in circulation is exposed to both plasma fibrinogen and plasminogen, we decided to study the combined anti-adhesive effect of these proteins on cell adhesion, while expecting an additive result. However, their effect was not additive. Surprisingly, fibrinogen markedly decreased the anti-adhesive effect of plasminogen. Utilizing U937 monocytic cells, a cell line commonly used for studying the assembly of the plasminogen activating system (Felez, Chanquia, Levin, Miles, & Plow, 1991; Gyetko, Shollenberger, & Sitrin, 1992; Kilpatrick et al., 2006), we have shown that fibrinogen actually interferes with the activation of fibrin-bound plasminogen and thus with its proteolytic anti-adhesive effect.

MATERIALS AND METHODS

Materials. Human plasminogen, plasmin, thrombin, and fibrinogen (depleted of fibronectin and plasminogen) were obtained from Enzyme Research Laboratories (South Bend, IN). Tissue-type plasminogen activator (t-PA) was purchased from Calbiochem (Darmstadt, Germany) and urokinase-type plasminogen activator (u-PA) was obtained from Calbiochem (San Diego, CA). Fibrinogen labeled with ^{125}Iodine using Pierce Iodination Beads (Thermo Scientific, Rockford, IL), was dialyzed against phosphate-buffered saline (PBS) and stored at −20 °C. Fibrinogen and plasminogen concentrations were determined based on their extinction coefficients, 1.56 and 1.7 at 1 mg/mL, respectively. Fibrinogen conjugated with Alexa Fluor® 488 and the reagents for labeling plasminogen with the Alexa Fluor® 647 dye were purchased from Invitrogen (Carlsbad, CA). Bovine serum albumin, phenylalanyl-L-prolyl-L-arginine chlormethyl ketone (PPACK) acid, and polyvinylpyrrolidone (PVP) were obtained from Sigma Aldrich (St Louis, MO). The chromogenic plasmin substrate D-Val-L-Leu-L-Lys *p*-nitroanilide hydrochloride (S-2251) was purchased from Chromogenix Diapharma Group (Franklin, OH). Calcein AM was purchased from Molecular Probes (Eugene, OR).

Preparation of fibrin gels. Fibrin gels were formed in siliconized wells of 96 well-format strips or 12 multiwell plates by mixing 60 μL or 250 μL, respectively, of 1.5 mg/mL fibrinogen in Hank's balanced salt solution (HBSS) with 0.25 U/mL thrombin for 2 h at 22 °C. Thrombin activity was quenched by incubation with 50 μM PPACK and the

gels were extensively washed with PBS. In selected experiments, fibrin gels were prepared from ^{125}I-labeled fibrinogen to incorporate a total of 1.5–2.0 × 10^5 cpm/gel.

Cell adhesion assays. U937 monocytic cells were obtained from ATCC and cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were labeled with 5 μM Calcein-AM for 30 min, washed twice with HBSS + 0.1% BSA. Adhesion assays were performed as previously described (Lishko, Burke, & Ugarova, 2007). Briefly, 100 μL of calcein-labeled U937 cells ($5x10^5$ /mL) in HBSS + 0.1% BSA were added to 96-multiwell format plate and incubated for 30 min at 37 °C. Next, non-adherent cells were removed by multiple washing with PBS (pH 7.4). Fluorescence intensity was measured in a CytoFluorII fluorescence plate reader (Applied Biosystems, Foster City, CA). To study the effect of fibrinogen on U937-mediated plasminogen activation, different concentrations of fibrinogen were mixed in with the cells. Alternatively, fibrin gels were incubated with various concentrations of soluble fibrinogen or plasminogen for 15 min at 22 °C, after which fibrinogen solutions were removed, fibrin gels washed and cells added to the wells. Data are expressed as a percentage of adherent cells.

Detection of plasminogen activation and plasmin production. Activation of plasminogen and generation of plasmin were determined by either measuring fibrin degradation products or plasmin amidolytic activity using the chromogenic substrate S-2251. To measure fibrin degradation products, fibrin gels were prepared from ^{125}I fibrinogen in 12-well format plates; 50–100 μg/mL plasminogen (0.25 mL) was

incubated with gels for 15 min at 22 °C. Gels were washed with PBS to remove unbound plasminogen, followed by the addition of U937 cells ($6x10^5$ /gel). After incubation for a specified period at 37 °C, the radioactivity of the supernatants containing fibrin degradation products was counted. The plasmin amidolytic activity generated by U937 cells adherent to immobilized plasminogen was also measured. Briefly, wells of 48-well Costar plates were coated with 100 μg/mL plasminogen, post-coated with 1% PVP, and washed with PBS. Different concentrations of U937 cells were added the wells and incubated for 30 min at 37 °C. Adherent cells were then removed, and the S-2251 color substrate was added to the wells (400 μL, 0.75 μM) and incubated for 2 h at 37 °C.

Confocal fluorescence microscopy. Fibrin gels were prepared in 50-mm Fluorodish™ dishes (World Precision Instruments, Sarasota, FL) by mixing 1.5 mg/mL fibrinogen in Hank's buffer with 0.25 U/mL thrombin. After 2 h incubation at 22 °C, thrombin activity was quenched by PPACK and fibrin gels were washed with PBS. 500 μL of 1 mg/mL Alexa 488-labeled fibrinogen was added to the gels with or without 100 μg/mL Alexa 647-labeled plasminogen. Adsorption of the fluorescently labeled proteins and their distribution on the surface and in the bulk of fibrin gels was observed with a 40x objective using a set-up based on a MicroTime200 Fluorescence lifetime microscope (PicoQuant, Berlin, Germany) and an Olympus IX-71 inverted microscope (Center Valley, PA), as previously described (Schulz et al., 2010).

RESULTS

The anti-adhesive effects of combined fibrinogen and plasminogen are not additive. Previously, we demonstrated that adsorption of either fibrinogen or plasminogen on the surface of fibrin gels dramatically reduces adhesion of platelets and leukocytes (Lishko, Burke, & Ugarova, 2007; Lishko et al., 2010). As shown in Fig. 7A, 1 mg/mL fibrinogen and 0.1 mg/mL plasminogen inhibited adhesion of U937 cells by ~ 70% and 90%, respectively. To investigate the possibility that the simultaneous addition of these proteins can produce a synergistic effect, fibrin clots were incubated with fibrinogen and plasminogen solution at lower concentrations to produce ~ 50% inhibition, and then adhesion of U937 cells to fibrin was examined. Adding both fibrinogen and plasminogen to fibrin gels did not result in an additive anti-adhesive effect (Fig. 7B). Moreover, a different effect was observed when fibrinogen, plasminogen or both proteins were added to U937 cells that have already been adherent to uncoated fibrin gels. While fibrinogen did not affect cell adhesion, apparently due to its inability to break the established cellular integrin-fibrin bonds, plasminogen continued to exert an anti-adhesive effect. This indicates that the generated plasmin was capable of digesting fibrin, resulting in the destabilization of the cell-fibrin attachment sites and loss of adhering cells (Fig. 7C). However, when the same concentrations of fibrinogen and plasminogen were mixed and added together, the inhibitory effect of plasminogen on cell adhesion was reduced compared with that of plasminogen alone (Fig. 7C). The latter result suggested that fibrinogen might be targeting and interfering with either the process of plasminogen activation or the proteolytic activity of the generated plasmin. In the next set of experiments, we examined these two possibilities.

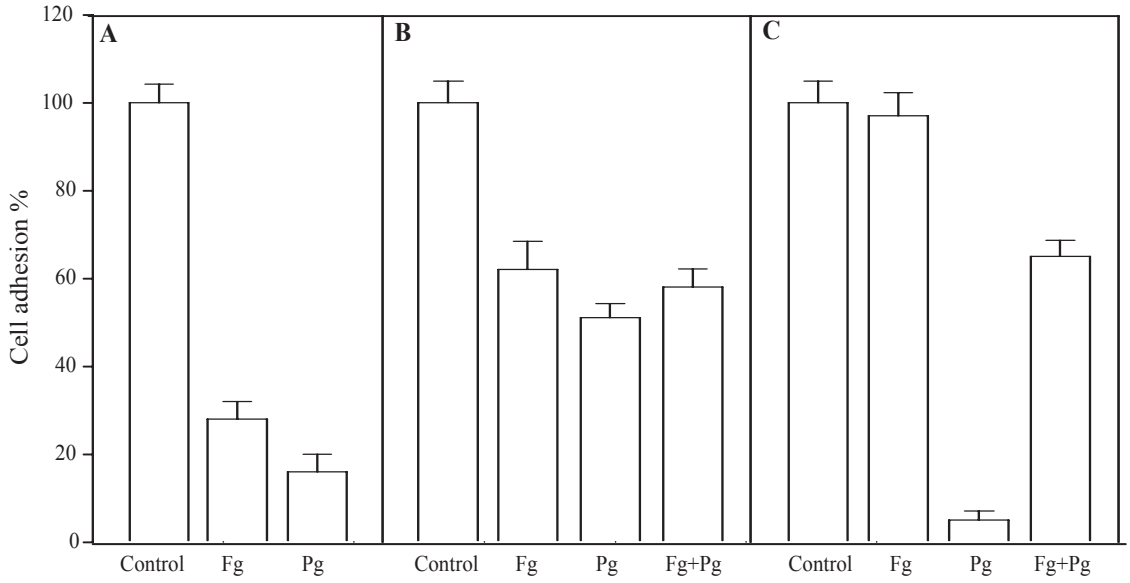


Figure 7. Effect of fibrinogen (Fg) and plasminogen (Pg) on adhesion of U937 cells to fibrin clots. **(A)** Fibrin gels were incubated with 1 mg/mL Fg or 100 μg/mL Pg for 30 min at 22 °C. Gels were briefly washed with PBS and calcein-labeled U937 cells were added. Adhesion of control U937 cells to untreated fibrin gels was assigned a value of 100% (control). **(B)** Fibrin gels were incubated with 50 μg/mL of fibrinogen, plasminogen or both for 30 min at 22 °C. The gels were washed with PBS and adhesion of U937 cells was measured. **(C)** U937 cells were allowed to adhere to untreated fibrin gels for 30 min at 37 °C, non-adherent cells were washed out and 1 mg/mL fibrinogen, 50 μg/mL plasminogen, or both were added to the adherent cells. After incubation for another 30 min at 37 °C, cell adhesion was determined by measuring fluorescence of adherent cells. Data are means ± SD of quadruplicate determinations from three to four experiments.

Fibrinogen interferes with activation of plasminogen by U937 cells. To determine whether fibrinogen intervenes in the activation of fibrin-bound plasminogen, U937 cells were mixed with different concentrations of fibrinogen and then added to fibrin gels with bound-plasminogen. As shown in Fig. 8A, fibrinogen blocked the formation of fibrin degradation products by the generated plasmin. This effect was dose-dependent and at 2 mg/mL, fibrinogen produced ~ 85% inhibition of plasmin activity as determined by the measurement of ^{125}I-labeled fibrin degradation products released in solution. Under the same conditions, release of fibrin degradation products in the absence of plasminogen was negligible; this suggested that plasmin-mediated fibrinolysis by U937 cells was superior to their non-plasmin dependent effects. Furthermore, addition of BSA to U937 cells, at a final concentration, which on a molar basis exceeds that of fibrinogen, did not affect cell adhesion to fibrin or generation of fibrin degradation products. Furthermore, It has been reported that fibrinogen can directly inhibit plasmin amidolytic activity (Higazi & Mayer, 1990). To assess the possibility that plasmin generated from fibrin-bound plasminogen is a target of fibrinogen, we examined the effect of fibrinogen on fibrin surface-bound plasmin. Fibrin gels were incubated with plasmin (10 U/mL) for 1 min at 4 °C and then extensively rinsed to remove any non-bound plasmin. Fibrinogen (1.5 mg/mL) was added to the plasmin-bound fibrin gels and the released radioactivity of solubilized fibrin was measured. Soluble fibrinogen did not influence the amount of fibrin degradation products released from the gels (Fig. 8B). The lack of a direct influence of fibrinogen on plasmin activity suggests that fibrinogen interferes with the process of U937 cell-mediated plasminogen activation and conversion into plasmin.

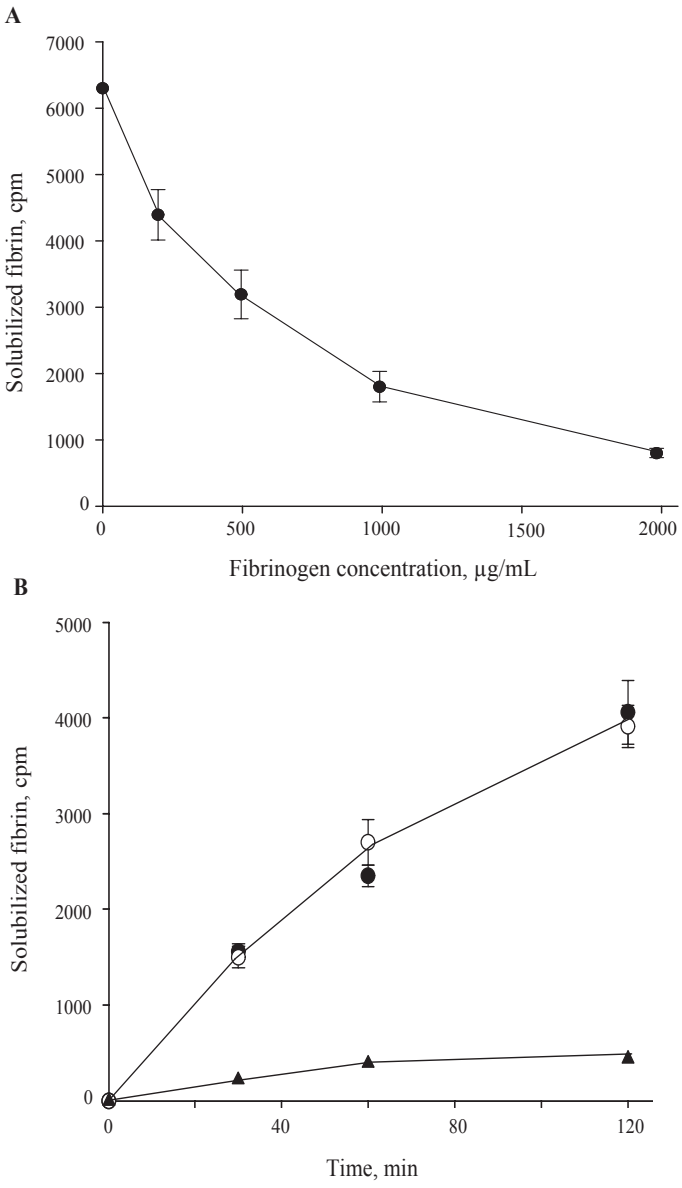


Figure 8. Soluble fibrinogen (Fg) inhibits activation of fibrin-bound plasminogen (Pg) by U937 cells. **(A)** Fibrin gels prepared from ^{125}I-labeled fibrinogen were treated with PPACK to quench thrombin activity. Total radioactivity was 174000± 3200 cpm/gel. Fibrin gels were incubated with 100 µg/mL Pg for 10 min at 22 °C, and then non-bound Pg was washed out. Suspensions of U937 cells (6×10^5) containing different concentrations of Fg were incubated with the gels for 30 min at 37 °C. Pg activation was detected by the solubilized radioactivity (cpm). Radioactivity dissociated from gels without added Pg (220±130 cpm) was subtracted. **(B)** Fibrin gels with bound plasmin (10 U/mL) were incubated with 1.5 mg/mL Fg (•) or buffer only (○) at 37 °C and the solubilized radioactivity was measured at the indicated time points. Solubilized radioactivity of gels without bound plasmin is also shown (⏏). Data are means ± SD of quadruplicate determinations from three experiments.

In addition to U937 cells, plasminogen activation and the generation of plasmin is also mediated by two physiological plasminogen activators: tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA). t-PA, the primary plasminogen activator in the vasculature, activates fibrin-bound plasminogen involved in fibrin dissolution in the circulation (E. Plow et al., 2001; Fay, Garg, & Sunkar, 2007; Tsurupa & Medved, 2001). In contrast, u-PA activates cell-bound plasminogen via binding to a specific cellular receptor, u-PAR, which then contributes to clot lysis (Longstaff, Clough, & Gaffney, 1992; Fleury, Lijnen, & Angles-Cano, 1993). Therefore, we sought to examine the effect of fibrinogen on the activation of fibrin-bound plasminogen by t-PA and u-PA (in the absence of cells). Plasminogen was bound to the surface of fibrin gels which were then incubated with either 10 ng/mL t-PA or 10 U/mL u-PA with and without fibrinogen for 2 h at 37 °C. The solubilized radioactivity, as a measure of plasmin activity, was determined. At 1.5 mg/mL, the concentration that inhibited the U937 cell-mediated plasmin generation by > 80%, fibrinogen did not have any significant effect on the activation of fibrin-bound plasminogen by both t-PA and u-PA as detected by the plasmin-mediated fibrin solubilization (Fig. 9). Altogether, the data shows that fibrinogen affects neither the fibrin surface-bound plasmin fibrinolytic activity nor the activation of fibrin surface-bound plasminogen by t-PA and u-PA. Rather, it interferes with the process of activation of fibrin-bound plasminogen by transiently adherent cells and, hence, exerts its effect at the cell-fibrin surface interface.

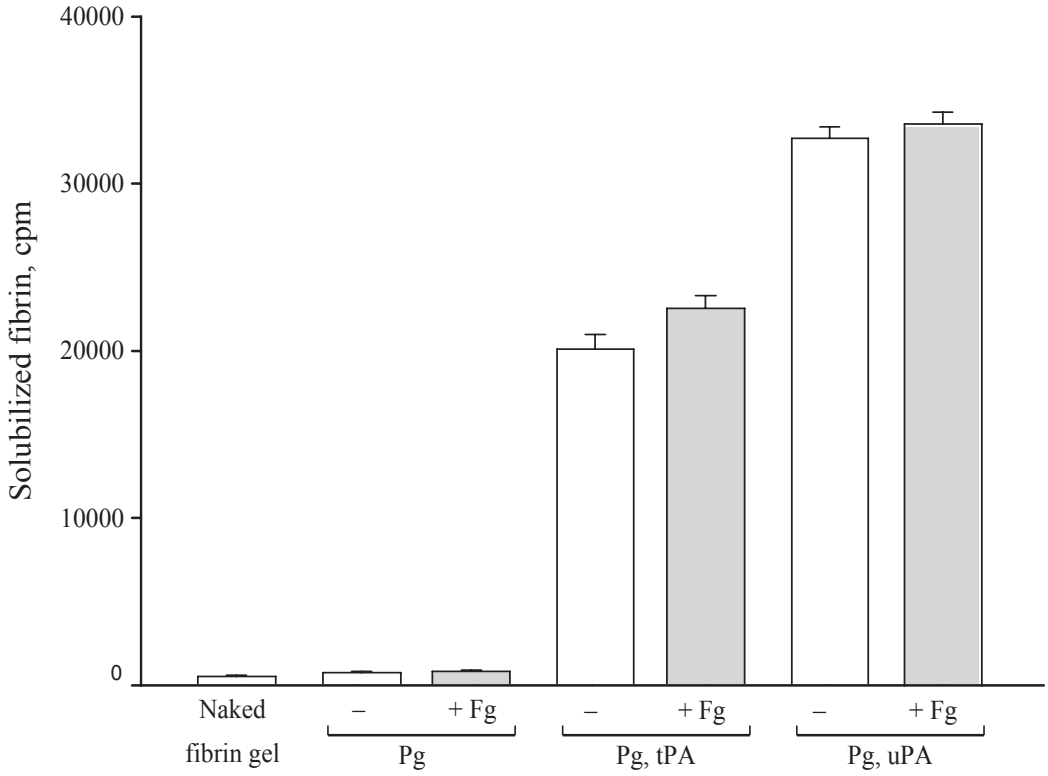


Figure 9. Soluble fibrinogen (Fg) does not affect activation of fibrin-bound plasminogen (Pg) by t-PA and u-PA. Fibrin gels prepared from ^{125}I-labeled fibrinogen were incubated with 50 µg/mL Pg for 15 min at 22 °C. Non-bound Pg was washed out with PBS. Solutions containing t-PA (20 ng/mL) or u-PA (10 U/mL) with (grey bars) or without (open bars) 1.5 mg/mL Fg were added to the gels and the solubilized radioactivity was measured after 2h incubation at 37 °C. Controls included gels without bound-Pg and gels with bound-Fg only. Data are means ± SD of triplicate determinations from three experiments.

Fibrinogen accumulates in a thin superficial layer of a fibrin gel and blocks activation of plasminogen by U937 cells. In our previous studies, we demonstrated that adsorption of fibrinogen on numerous hard surfaces, including plastic, mica and glass, results in the formation of a non-adhesive multilayer matrix (Lishko, Yermolenko, & Ugarova, 2010; I. S. Yermolenko et al., 2010). Also, addition of soluble fibrinogen and its adsorption onto the surface of fibrin clots results in a dramatic decrease in cell adhesion (Lishko, Burke, & Ugarova, 2007; Lishko, Yermolenko, & Ugarova, 2010), thus suggesting that fibrin may somehow induce aggregation of the fibrinogen molecules.

However, the accumulation of fibrinogen in a thin superficial layer of a fibrin clot has not been previously observed. In order to investigate the processes occurring at the fibrin surface, we initially examined the kinetics of fibrinogen adsorption using a fluorescein-labeled tracer. Briefly, fibrin gels were incubated with a 1 mg/mL Alexa 488-labeled fibrinogen for various time periods. The gels were then washed several times with PBS (pH 7.4) so that the binding is stopped. As shown in Fig. 10, fibrinogen was bound to the fibrin gels in a time-dependent manner. Notably, a significant amount of fibrinogen was associated with fibrin within 30 min. We also examined the kinetics of binding of ^{125}Iodine-labeled fibrinogen, which produced a similar pattern of adsorption (Fig. 10). In accordance with the deposition of fibrinogen was a rapid decrease in U937 cell adhesion (Fig. 10).

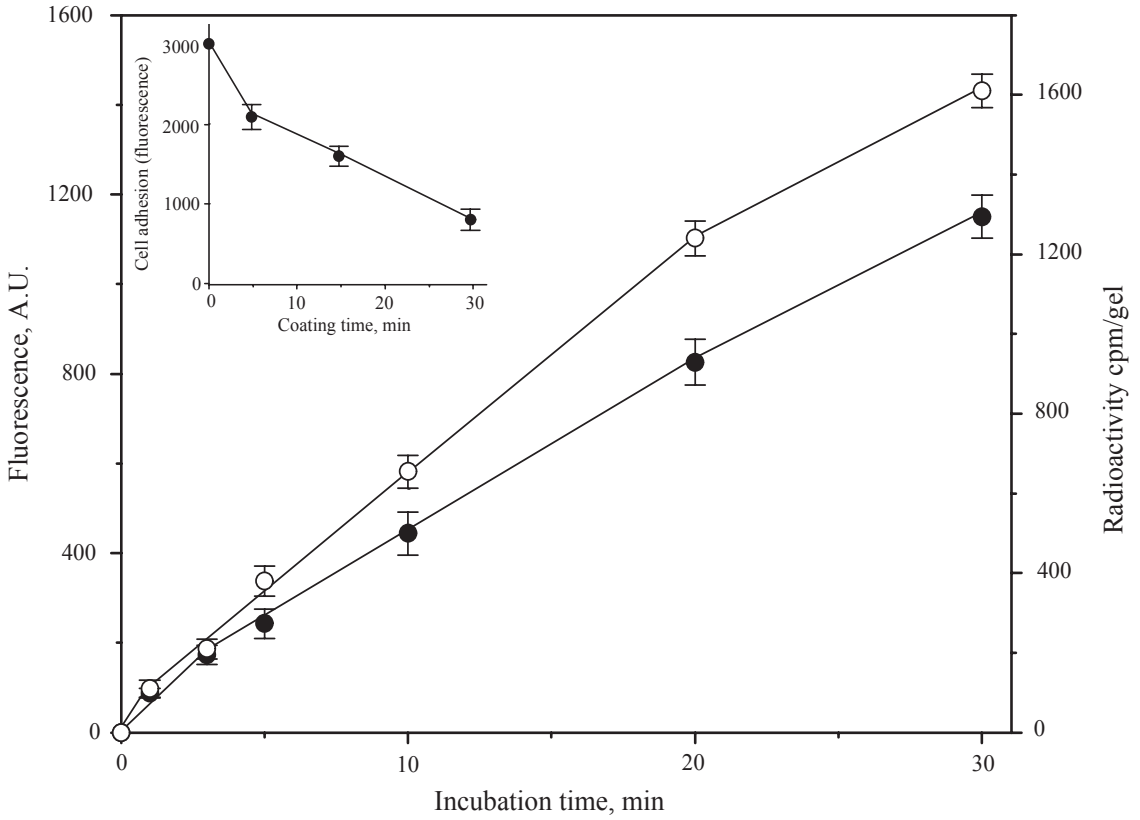


*Figure 10. Kinetics of fibrinogen (Fg) adsorption to fibrin gels***.** Fibrin gels were incubated with 1 mg/mL fibrinogen containing Alexa 488-labeled Fg (○) or ^{125}I-labeled Fg (•) for various time points. Fibrin gels were washed to remove non-bound Fg and then fluorescence or radioactivity was measured. Data are means ± SD of quadruplet determinations from two to three Inset: adhesion of U937 cells to fibrin gels incubated with 1 mg/mL fibrinogen.

Furthermore, we closely examined the spatial distribution and adsorption of fibrinogen, mainly on the surface of fibrin gels using confocal fluorescence microscopy. 1 mg/mL of Alexa 488-labeled fibrinogen was added atop the fibrin gels, incubated for various time points including 2.5 min, 5 min, 10 min, and 30 min, and its accumulation at the gel's surface was monitored. Adsorption of fibrinogen on the fibrin gel surface was observed as early as 2.5 min and continued to increase with time. Quantification of the fluorescent signal at the surface and throughout the bulk of the gel indicated that the amount of deposited fibrinogen reached a maximal level after the 30 min incubation (Fig. 11A). However, the spatial distribution of fibrinogen across the gel had a non-uniform pattern, with a 2-fold higher fluorescence intensity detected as a thin layer (~ 10 μm thick fluorescent band) present at the fibrin surface. Selected fibrin gels with bound fibrinogen were extensively washed with PBS to remove any weakly- or non-bound fibrinogen. Washing the gels resulted in a more pronounced difference in fluorescence intensity, which was ~ 15-fold higher at the surface than inside the gel. This finding implies that a significant portion of the added fibrinogen is truly adsorbed only at the surface of the fibrin gel (Fig. 11B).

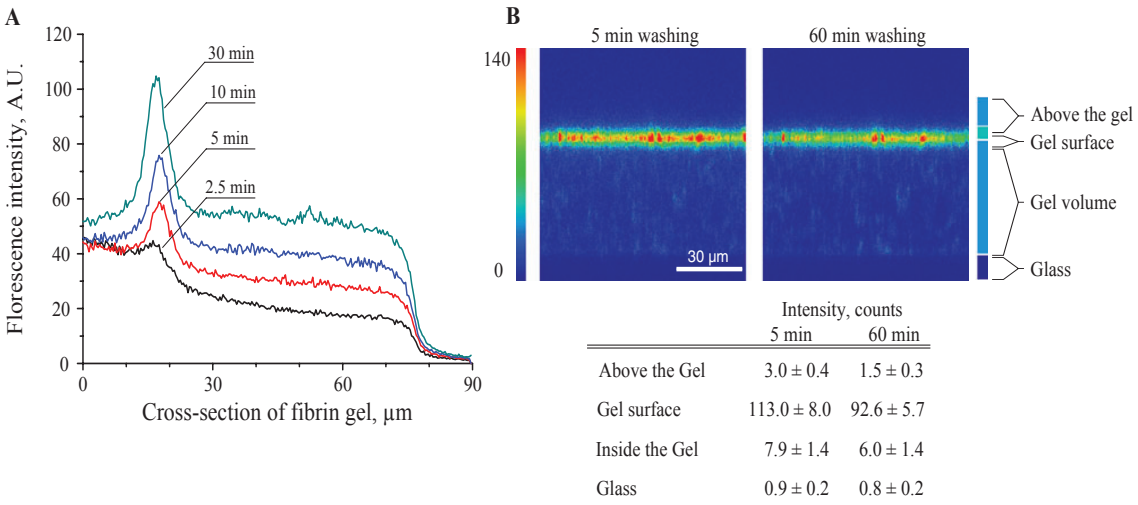


Figure 11. Fibrinogen (Fg) accumulates in a thin superficial layer of fibrin clots. **(A)** Fibrin gels prepared in 50 mm Fluorodish™ glass-bottom dishes were incubated with 1 mg/mL Alexa-488 labeled Fg for 2.5, 5, 10 and 30 min. Fluorescence intensity of fibrin gel cross-sections is measured and averaged by 64 consecutive pixels. **(B)** Confocal images of a representative gel after 2h incubation with 1 mg/mL Alexa 488-labeled Fg and washing with PBS for 5 and 60 min at 22 °C. A color scale bar on the left represents the relative intensity in photons/pixel, with the lowest intensity corresponding to the glass surface. The table below shows the distribution of fluorescence intensity in the solution above the gel, at the gel surface, inside the gel, and inside the glass in photons/pixel. The intensity data shown are the averaged areas.

Plasminogen can also bind to fibrin and accumulate as a superficial layer on the surface of fibrin gels (Sakharov & Rijken, 1995; Sakharov, Nagelkerke, & Rijken, 1996). This, we believe, may interfere with the occupancy and adsorption by fibrinogen. To test this possibility, we simultaneously added Alexa 488-labeled fibrinogen (1 mg/mL) and Alexa 647-labeled plasminogen (0.1 mg/mL) to the fibrin gel and their adsorption at the surface was detected using confocal fluorescence microscopy. As shown in Fig. 12A, both proteins accumulated in the superficial layer of the gel. Quantification of fluorescence intensity, after washing the gels for 60 min, revealed that a significant amount of both proteins remained associated at the surface with a detectable amount of

plasminogen also found inside the gel. Fibrinogen and plasminogen had ~ 18 and 3-fold higher fluorescence intensity, respectively, on the surface than inside the gel. These data suggests that binding of plasminogen is largely independent of fibrinogen deposition and adsorption.

Next, we demonstrated that fibrin surface-bound fibrinogen was responsible for blocking or interfering with the U937 cell-mediated plasminogen activation and plasmin generation. To do so, ^{125}I-labeled fibrin gels were initially incubated with plasminogen and then with varying concentrations of fibrinogen, followed by washing to remove any non-bound proteins and finally incubated with U937 cells. As shown in Fig. 12B, transiently adherent U937 cells gradually lost their ability to activate bound plasminogen and generate plasmin, which is measured in the form of solubilized radioactivity, with the increasing concentrations of adsorbed fibrinogen. 50% inhibition of U937 mediated-plasmin generation was detected at fibrinogen concentration of ~ 0.45 mg/mL, whereas 90% inhibition was attained at the concentration of 2 mg/mL. These experimental conditions differ from those described in Fig. 8, in that U937 cells were added to fibrin gels with already surface-bound fibrinogen and without any additional soluble fibrinogen. There is a possibility that bound fibrinogen could dissociate from the surface and when cells are added a new equilibrium is established between the soluble and surface-bound fibrinogen. However, only small amounts of fibrinogen (~ 12 μg/mL; 35 nM) were found to dissociate from the clot's surface, which seems insufficient to block monocyte receptors which bind fibrinogen with a dissociation constant (*K*d) in the μM range (Felez, Chanquia, Levin, Miles, & Plow, 1991). Therefore, the inability of U937 cells to activate plasminogen may be attributed to fibrinogen bound with the surface of fibrin gels.

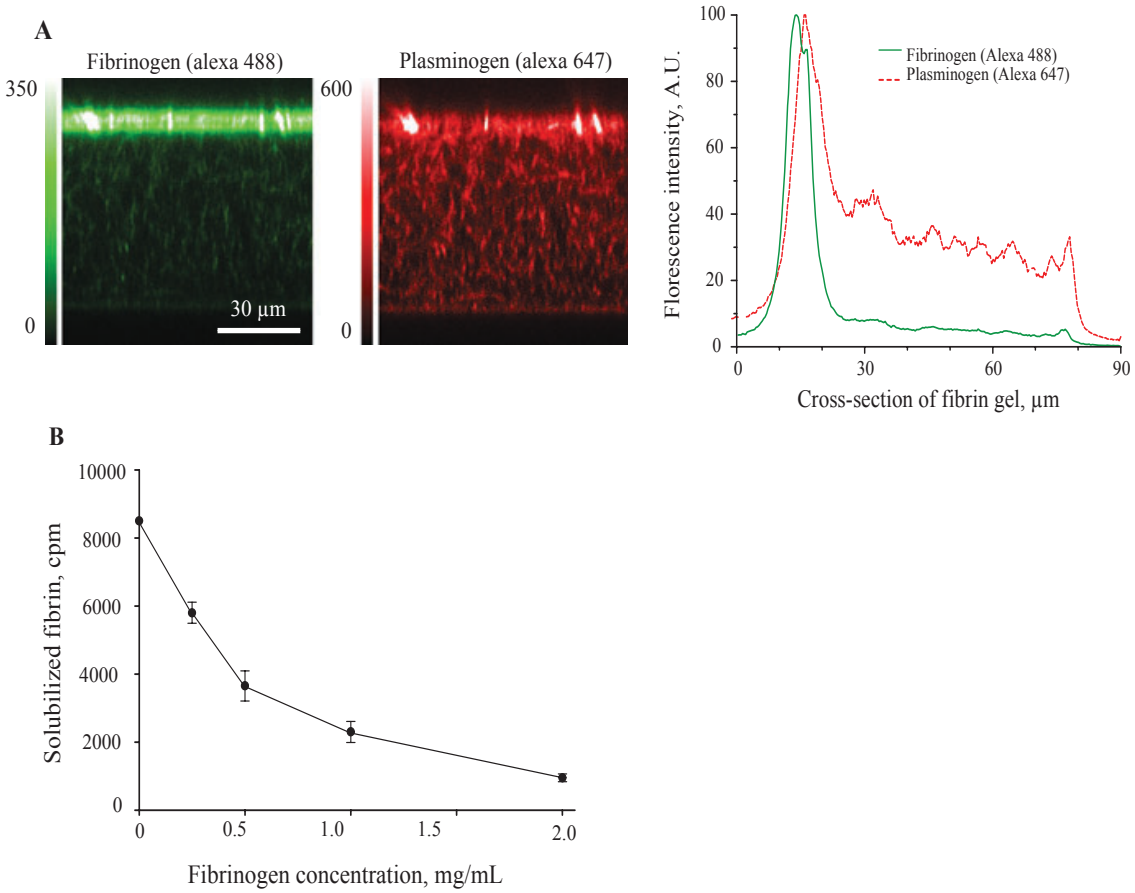


Figure 12. Fibrinogen (Fg) deposited at fibrin clot surface prevents activation of fibrin-bound plasminogen (Pg) by U937 cells. **(A)** Fibrin gels were incubated with 1 mg/mL Alexa 488-labeled Fg and 100 µg/mL Alexa 647-labeled Pg for 30 min at 22°C. Confocal images were made 1h after washing of non-bound proteins. Shown to the right of the images are the corresponding fluorescence intensities of fibrin gel cross-sections (averaged by 64 consecutive pixels). **(B)** 100 µg/mL Pg was bound to the surface of fibrin gels (182000 ± 6100 cpm/gel) and then various concentrations of Fg were added to the gels for 15 min at 22 °C. Following washing, U937 cells (6×10^5) were added to the gels and incubated for 60 min at 37 °C, after which solubilized radioactivity was measured. The result shown is representative of three independent experiments.

Fibrinogen masks the access of U937 cells to surface-immobilized plasminogen. A requirement for cells to activate fibrin surface-bound plasminogen is obviously that they come in contact with it. To explore the underlying mechanism by which fibrinogen blocks activation of plasminogen at the fibrin-cell surface interface, we performed the next set of experiments with plasminogen immobilized onto plastic. For technical reasons, fibrin gels with bound plasminogen could not be used; this is because as soon as the cells engage and activate plasminogen, the generated plasmin will degrade the fibrin surface causing cell detachment. Therefore, plasminogen was immobilized onto a hard surface as plastic to definitively examine the effect of fibrinogen and the mechanism by which it blocks plasminogen activation occurring at the fibrin-cell surface interface. In agreement with previous studies (Lishko, Novokhatny, Yakubenko, Skomorovska-Prokvolit, & Ugarova, 2004), the immobilized plasminogen supported strong cell adhesion (Fig. 13A). Moreover, the adherent cells were able to activate plasminogen; however, the generated plasmin did not cause cell detachment (Fig. 13A). Furthermore, treating plasmin with PPACK had no effect on cell adhesion, confirming that both active and inactivated immobilized plasmin support adhesion. These results are contrary to the results obtained using fibrin-bound plasminogen where its activation causes cell detachment. A significant decrease in cell adhesion was observed when fibrinogen was added and bound to the immobilized plasminogen (Fig. 13B). This indicates that plasminogen is capable of interacting with fibrinogen, which is in agreement with previous studies (Varadi & Patthy, 1983; Tsurupa & Medved, 2001). Additionally, fibrin-bound fibrinogen resulted in a pronounced decrease in plasminogen activation and thus plasmin generation, as measured by the plasmin chromogenic

substrate (Fig. 13C). Overall, these results suggest that binding of fibrinogen blocks access to the immobilized plasminogen, therefore preventing the plasminogen activators assembled on a cell's surface from engaging plasminogen and activating it.

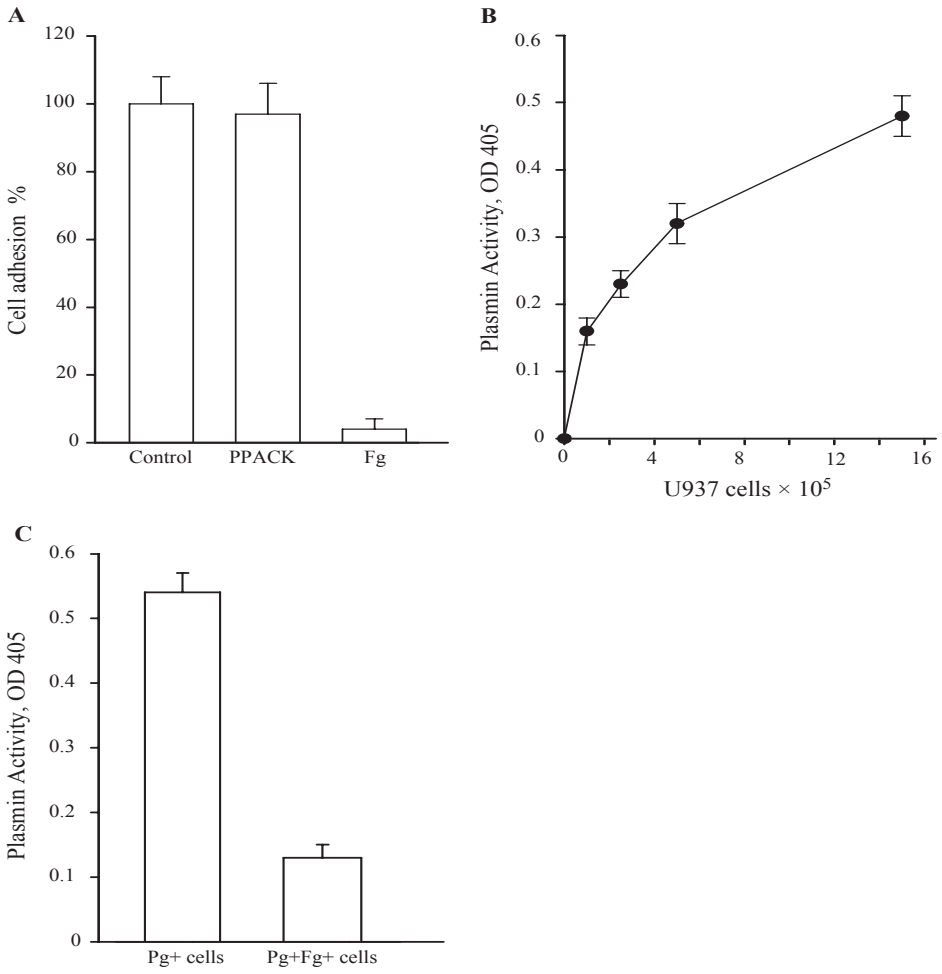


Figure 13. Adhesion of U937 cells to immobilized plasminogen (Pg) and its activation by adherent cells. **(A)** Wells coated with 10 μg/mL Pg, blocked with 1% PVP, followed by incubation with PBS (control) or 100 μg/mL Fg. Adhesion of calcein-labeled U937 cells (5×10^4) was measured in the presence or absence of PPACK. **(B)** Wells coated with 100 μg/mL Pg, post-coated with 1% PVP and different concentrations of U937 cells were added for 30 min at 37 °C. Adherent cells were removed and S-2251 was added and incubated for 2h at 37 °C. **(C)** 100 μg/mL Fg was added to wells pre-coated with 100 μg/mL Pg and incubated at 22 °C for 20 min. U937 cells (5×10^5) were added and incubated for 30 min at 37 °C. Adherent cells were removed and plasmin activity was measured using S-2251. Wells without added Fg served as a control.

DISCUSSION

In this study we examined the interplay between two anti-adhesive systems that operate at the surface of fibrin clots; the non-proteolytic fibrinogen-dependent and the proteolytic plasminogen-dependent system. The major finding of this study is that when fibrin clots are simultaneously exposed to plasma proteins fibrinogen and plasminogen, as it would occur in the circulation, their anti-adhesive effects are not additive. However, the deposited fibrinogen counteracts the anti-adhesive effect of plasminogen by masking it and preventing its activation by transiently adherent cells.

Previously, we demonstrated that adsorption of fibrinogen on various hard surfaces such as plastic, mica, and glass render them poorly adhesive. The mechanism underlying the anti-adhesive effect of fibrinogen arises from the formation of an extensible multilayered fibrinogen matrix. This matrix is incapable of transducing strong integrin-mediated mechanical forces, thus resulting in weak cell adhesion and subsequent cell detachment under flow (Lishko, Yermolenko, & Ugarova, 2010). Furthermore, we have also shown, using atomic force microscopy (AFM) and AFM-based force spectroscopy, that deposition of fibrinogen in the form of a molecular bilayer is sufficient to render surfaces non-adhesive (I. S. Yermolenko et al., 2010). Moreover, deposition of fibrinogen onto fibrin clots at increasing concentrations was found to dramatically reduce the adhesion of platelets and leukocytes (Lishko, Burke, & Ugarova, 2007; Lishko, Yermolenko, & Ugarova, 2010). However, it remained uncertain whether fibrinogen could accumulate and concentrate at the surface of a fibrin clot. As the characterization of fibrinogen adsorption on fibrin clots is not readily amenable to AFM, we investigated this process using confocal fluorescence microscopy.

Our results show, for the first time, that fibrinogen accumulates in a thin layer at the fibrin surface, where its concentration, even after extensive washing, remained several-fold higher than inside the gel (Fig. 11). Plasminogen, which is also known to accumulate in a superficial layer of a fibrin clot (Sakharov, Nagelkerke, & Rijken, 1996), was found to not interfere with the deposition of fibrinogen at the fibrin surface (Fig. 12). The intrinsic ability of fibrinogen to aggregate upon contact with various surfaces implies that it may accumulate at surface of fibrin clots in the form of a non-adhesive multilayer. The results show that in the absence of fibrinogen, plasminogen deposited at the clot surface is capable of exerting its potent anti-adhesive effect. This is because transiently adherent cells are able to engage the fibrin surface-bound plasminogen and activate it. The generated plasmin, in turn, degrades fibrin fibers at the cell-fibrin surface interface resulting in cell detachment (Lishko et al., 2010). In contrast, when fibrinogen begins to accumulate at the fibrin surface it gradually diminishes the plasminogen-dependent anti-adhesive effect.

The precise mechanism by which the simultaneous adsorption of fibrinogen and plasminogen at the clot's surface inhibits the cell-mediated plasminogen activation is not fully understood and will require further study. However, the simplest interpretation of the observed phenomenon is that fibrinogen deposits at the fibrin surface in the form of a multilayered fibrinogen matrix which masks the surface-bound plasminogen and precludes its physical contact with cells. Indeed, as shown in Fig. 13, overlaying fibrinogen atop the immobilized plasminogen apparently created a barrier between the plasminogen and cells, which blocked plasminogen activation and plasmin generation. It is likely that the use of high fibrinogen concentration in our experiments, to duplicate a ~

10:1 ratio of fibrinogen to plasminogen as in circulation, was a major factor in its dominating effect. Alternatively, the deposited multilayered fibrinogen matrix can potentially induce rearrangements in the interaction of $\alpha_M\beta_2$ (Mac-1) integrin, a major fibrinogen receptor on monocytes, with u-PAR, a surface receptor for u-PA. This is in agreement with previous studies demonstrating that $\alpha_M\beta_2$ forms a functional unit with u-PAR (Xue, Kindzelskii, Todd, & Petty, 1994; Simon et al., 1996), and both receptors cooperate during adhesion of activated monocytes to fibrinogen (35). Determining the exact mechanism by which the multilayered matrix may have a role in influencing the signaling and/or the proteolytic functions of the $\alpha M\beta_2$/u-PAR-u-PA system will be an important direction for future investigations.

The ability of transiently adherent U937 monocytic cells, which are known to have a fully assembled plasminogen activation system on their surface, to activate fibrin-bound plasminogen has been previously reported (Lishko et al., 2010), and is in accordance with studies that used THP-1 monocytic cells (Dejouvencel et al., 2010). When performing assays using both of these cells and in the absence of fibrinogen, U937 and THP-1 cells were able to efficiently activate fibrin-bound plasminogen. Based on this finding, it was proposed (Dejouvencel et al., 2010) that activation of fibrin-bound plasminogen by u-PA-bearing cells through an interfacial mechanism (i.e. a process involving contact of two surfaces) might represent a new mechanism of plasmin generation. The finding of this study, that fibrinogen blocks activation of fibrin-bound plasminogen, brings the universality of the proposed model into question as it suggests that the relationship between fibrin and monocytes may vary in different locations. Thus, plasminogen activation occurring at the interface between the surface of fibrin clots and

adherent cells may occur only in locations where fibrinogen is not expected to be deposited at the clot surface and interfere with plasminogen activation. Conversely, uPA-bearing monocytes that migrate to the clot's interior through the vessel wall (McGuinness et al., 2001) may directly activate fibrin-bound plasminogen because the interior is not exposed to blood flow and thus is depleted of any soluble fibrinogen.

It is important to note that fibrin-bound plasminogen can be activated not only by U937 monocytic cells but also by freshly isolated peripheral blood monocytes. However, in contrast to U937 cells that not only produce their own u-PA but also bind it via u-PAR, freshly isolated blood monocytes do not contain any detectable levels of u-PA on their surface (Gyetko, Shollenberger, & Sitrin, 1992). Furthermore, these isolated monocytes have no detectable levels of t-PA on their surface. This might be to due to the very low physiological levels of functional t-PA (20 pM) as well as the low binding capacity of monocytes to t-PA with a K_d in the μM range (Felez, Chanquia, Levin, Miles, & Plow, 1991). Since the levels of both activators become substantially elevated only during vascular injury and inflammation, isolated monocytes must be supplemented with either exogenous u-PA or t-PA in order to activate fibrin-bound plasminogen. Thus, it appears that both U937 monocytic cells and isolated monocytes have limitations as experimental models. Additional work will be required to fully explore the effect of fibrinogen on activation of fibrin-bound plasminogen by monocytes and other blood cells, such as platelets, both *in vitro* and *in vivo*.

Although fibrinogen and plasminogen both can exert potent anti-adhesive effects at the fibrin clot surface, there seems to be a clear superiority of the fibrinogen-dependent system. Therefore, it remains uncertain under which conditions the plasminogen-

dependent system may play a significant role. One possibility for such disparity is the heterogeneity of the fibrin clot surface. A recent study in living mice has shown that as the thrombus grows, its surface, consisting primarily of platelets, changes to that composed of fibrin. The fibrin surface displayed variable intensities of the fibrin-, platelet-, and fibrinogen-specific fluorophores, indicating uneven surface coverage with fibrinogen (Cooley, 2011). Such surface heterogeneity implies that fibrinogen and plasminogen may potentially accumulate at different regions of the clot surface. Another possibility is that pathological conditions may alter the contribution of fibrinogen and/or plasminogen to the anti-adhesive effect. Further investigations on the spatial distribution and localization of fibrinogen and plasminogen on the surface of clots, both *in vitro* and *in vivo,* will be necessary to fully comprehend the roles and contributions of the two anti-adhesive systems.

Despite the fact that fibrin is a highly adhesive substrate for both platelets and leukocytes *in vitro*, the fibrin surface of stable hemostatic thrombi in circulation does not effectively support adhesion of these cells. The paradox as to why fibrin in the circulation remains non-thrombogenic has yet to be addressed. We proposed that a reason for such non-thrombogenic nature is the ability of fibrin to bind both plasma proteins fibrinogen and plasminogen, resulting in the assembly of potent anti-adhesive systems that guard the clot's surface from excessive cell accumulation. These protective mechanisms, we believe, may satisfy a dual purpose. First, it may prevent the continuous accumulation of platelets, thereby limiting thrombus growth beyond that needed for stabilization. Second, it could limit the uncontrollable recruitment of leukocytes, whose adhesion is concurrent with the release of proteolytic enzymes, therefore protecting the thrombus from

premature lysis and allowing early hemostasis to proceed unchallenged. The efficiency of both the fibrinogen- and plasminogen-dependent anti-adhesive processes observed *in vitro* as well as the supporting evidence from *in vivo* studies implicates their physiological relevance. Altogether, the findings of this study revealed not only the significant contribution of fibrinogen and plasminogen to normal hemostasis but also the intricate relationship between the two anti-adhesive processes. The existence of such surface-mediated control of thrombus growth and stability may represent a previously unrecognized aspect of hemostasis and set the direction for future research.

CHAPTER 3

DUAL ROLE OF PLATELETS IN THROMBUS FORMATION

ABSTRACT

In this study, we demonstrate that platelets pre-bound to a plasminogen activator can activate fibrin-bound plasminogen through an interfacial mechanism, i.e. when the components of the plasminogen activation system are assembled on different surfaces. Specifically, tissue plasminogen activator (t-PA) or urokinase plasminogen activator (u-PA) pre-bound to one population of platelets is able to activate plasminogen pre-bound to a separate pool of platelets. This suggests that platelets can activate either platelet- or fibrin-bound plasminogen into plasmin, and therefore could use this fibrinolytic activity during adhesion to fibrin clots. However, it is uncertain whether this anti-adhesive mechanism is functional when encountering platelet-rich fibrin clots, since platelets are to known to secrete high levels of plasminogen activator inhibitor-1 (PAI-1) during clot formation. To address this question, we have compared the fibrinolytic activity of t-PA- or u-PA-treated surface-adherent platelets with that of fibrin-incorporated platelets. While fibrinolysis by platelets inside the fibrin gel was completely inhibited, adherent platelets retained their ability to activate plasminogen and degrade fibrin. Also, we found that fibrin-incorporated platelets are able to inhibit the fibrinolytic activity of fibrin-incorporated but not fibrin-adherent U937 monocytic cells. Confocal microscopy and Western blot analyses showed that by contrast with fibrin-incorporated platelets, which released substantial amounts of PAI-1; no PAI-1 was secreted from surface-adherent platelets. These data may explain the well-known but hitherto unexplained capacity of

platelets to either promote or inhibit fibrinolysis depending on their location within the thrombus. Thus, platelets inside the clot, via the release of PAI-1, block fibrinolysis aiding in thrombus integrity. In contrast, platelets transiently contacting fibrin on the surface of clots are pro-fibrinolytic, enabling the anti-adhesive mechanism, which prevents their excessive accumulation and, consequently vessel occlusion.

INTRODUCTION

The process of hemostatic thrombus formation in response to vascular trauma is a highly regulated event. This process helps terminate blood loss and facilitates tissue healing by ensuring that the forming thrombus is strong enough to secure the breach but not excessively robust to cause blood vessel obstruction and subsequent thrombosis. Upon blood vessel injury, circulating platelets serve to form a platelet plug. Platelets adhere to the exposed sub-endothelial extracellular matrix, where they become activated and aggregated. The initial accumulation of platelets is followed by a thrombin-mediated conversion of plasma fibrinogen into fibrin. The continued deposition of fibrin results in the formation of an extensive fibrin network that serves as a scaffold necessary to stabilize the entire thrombus and further seal up the breach (Baumgartner, 1973; Hattori et al., 1978; van Ryn et al., 2003; Beygui et al., 2006; S. Falati et al., 2002; Groves et al., 1982). Nevertheless, to limit thrombus growth at the site of vascular injury, both the coagulation and fibrinolytic systems are under strict regulation and act in concert to maintain control of thrombus formation, stability, and timely dissolution. Therefore, under physiological conditions the formed hemostatic seal gradually undergoes proteolytic digestion. This process involves conversion of insoluble fibrin into a soluble

degradation product as the blood vessel heals in order to maintain vasculature patency and normal blood flow (Rijken & Lijnen, 2009; Kotb, 2014).

Previously, we have identified two anti-adhesive mechanisms operating on the surface of fibrin clots that prevent excessive accumulation of blood cells, such as platelets and leukocytes (Lishko et al., 2007). Therefore, limiting thrombus growth beyond the site of injury. In particular, adsorption of fibrinogen and plasminogen to fibrin creates a mechanically unstable surface through non-proteolytic and proteolytic mechanisms, respectively, which limit integrin-mediated cell adhesion. The non-proteolytic process is based on the surface-induced self-assembly of fibrinogen molecules in the form of a multilayer matrix. This matrix is extensible and thus incapable of transducing strong mechanical forces through cellular integrins. On the contrary, the proteolytic process depends on the activation of fibrin surface-bound plasminogen by the plasminogen activation system assembled on the surface of transiently adherent blood cells. This is followed by the generation of plasmin, which degrades the fibrin surface, resulting in cell detachment under flow (I. S. Yermolenko et al., 2010; Lishko et al., 2010; Lishko, Yermolenko, Owaynat, & Ugarova, 2012; Endenburg et al., 1996; Kuijper, Gallardo Torres, van der Linden, et al., 1997; Fay & Owen, 1989).

Platelets are known to play a critical role in regulation of thrombus formation, stability, and dissolution. They are involved in the initial formation of the platelet/fibrin hemostatic plug and later help maintain thrombus integrity through the release of plasminogen activator inhibitors (PAIs). A major regulatory protein of the fibrinolytic system *in vivo* is type-1 PAI referred to as PAI-1, a single chain protein with a molecular weight of ~45 kDa. PAI-1 reacts rapidly with tissue plasminogen activator (t-PA) or

urokinase plasminogen activator (u-PA), either in solution or at the fibrin surface, resulting in the formation of very stable inactive PAI-1/PA complexes (Loskutoff, Sawdey, & Mimuro, 1989; Van Meijer & Pannekoek, 1995; Robbie, Bennett, Croll, Brown, & Booth, 1996; Cesarman-Maus & Hajjar, 2005). During adhesion to the surface of fibrin clots, platelets have been shown to enhance the activation and conversion of fibrin-bound plasminogen (Pg) into plasmin, the major fibrinolytic serine protease, in the presence of its activators, t-PA or u-PA, leading to fibrin clot degradation and solubilization through the proteolytic process (Rijken & Lijnen, 2009; Kotb, 2014). However, this proteolytic mechanism operating on the surface of the clot encounters two fundamental obstacles. First, platelets have been shown to be the main source of PAI-1 and contain high levels of active PAI-1, which is secreted from the alpha granules of activated platelets during fibrin clot formation, effectively inhibiting fibrinolysis (Erickson, Ginsberg, & Loskutoff, 1984; Kruithof, Tran-Thang, & Bachmann, 1986; Sprengers, Akkerman, & Jansen, 1986; E. F. Plow & Collen, 1981). Therefore, it is uncertain whether the proteolytic anti-adhesive mechanism is functional when platelets encounter platelet-rich fibrin clots. Second, the reaction of α2-antiplasmin, which is present in both platelets and plasma, with plasmin to form an enzymatically inactive complex is considered to be one of the fastest protein-protein reactions known (Wiman & Collen, 1978; Felez et al., 1991). How this proteolytic anti-adhesive mechanism may function in the presence of these two potent inhibitors, PAI-1 and α2-antiplasmin, needs further explanation.

To address our question, we chose to study two cell populations: platelets, the primary element involved not only in the formation of the hemostatic plug, but also in

regulation of thrombus growth and stability. By comparison, cultured U937 monocytic cells, which are known to have a fully assembled plasminogen activation system on their surface, represent an ideal cell model to study the process of plasminogen activation and fibrinolysis (Felez et al., 1991; Gyetko et al., 1992; Kilpatrick et al., 2006). Our data suggest that platelets can either promote or inhibit the process of fibrinolysis depending on their location within the thrombus.

MATERIALS AND METHODS

Materials. Human plasminogen (depleted of plasmin activity), plasmin, thrombin and fibrinogen (depleted of fibronectin and plasminogen) were purchased from Enzyme Research Laboratories (South Bend, IN). Tissue-type plasminogen activator (t-PA) was purchased from Calbiochem (Darmstadt, Germany) and urokinase-type plasminogen activator (u-PA) was obtained from Calbiochem (San Diego, CA). Fibrinogen labeled with ^{125}Iodine using Pierce Iodination Beads (Thermo Scientific, Rockford, IL), dialyzed against phosphate-buffered saline (PBS) and stored at - 20 °C. Fibrinogen and plasminogen concentrations were determined based on their extinction coefficients, 1.56 and 1.7 at 1 mg/mL, respectively. Bovine serum albumin, phenylalanyl-L-prolyl-L-arginine chlormethyl ketone (PPACK) acid and polyvinylpyrrolidone (PVP) were obtained from Sigma Aldrich (St Louis, MO). Diethylaminoethyl-Sephacel was obtained from Sigma Aldrich (San Louis, MO). The chromogenic plasmin substrate D-Val-L-Leu-L-Lys p-nitroanilide hydrochloride (S-2251) was purchased from Chromogenix Diapharma Group (Franklin, OH). Calcein-AM was from Molecular Probes (Eugene, OR). The mouse monoclonal anti-PAI-1 antibody was purchased from EMD Millipore

(Darmstadt, Germany). Alexa Fluor 488 dye was obtained from Life Technologies (Carlsbad, CA). Penicillin-streptomycin solution (10,000 I.U. Penicillin, 10,000 μg/ml Streptomycin) was purchased from Corning (Cellgro, Manassas, VA). Costar multi-well cell culture plates were bought from Corning Inc. (Corning, NY).

Preparation of fibrin gels. Fibrin gels were made in Surfasil-siliconized wells of 96-well format strips or 24 and 12-well format Costar plates by mixing 50-100 μL or 250 μL, respectively, of 1.5 mg/mL fibrinogen in Hank's balanced salt solution (HBSS) with 0.25 U/mL thrombin for 1 h at 22 °C. In selected experiments, thrombin activity was quenched by incubation with 50 μM PPACK. PPACK-treated fibrin gels were washed multiple times with PBS (pH 7.4). In other selected experiments, fibrin gels were prepared from ^{125}I-labeled fibrinogen to incorporate a total of 1.5–2.0x10^5 cpm/gel. All ^{125}I-labeled fibrin gels were extensively washed with PBS (pH 7.4) and released radioactivity was counted until it was minimal.

Platelet isolation. Blood was obtained via venipuncture from healthy volunteers who had not taken any aspirin in the previous two weeks. Briefly, the collected blood was anti-coagulated with acid citrate dextrose (ACD) in the presence of 2.8 μM prostaglandin E1 (PGE1). Platelets were obtained by differential centrifugation, followed by gel-filtration on Sepharose 2B-CL in calcium-free Tyrodes buffer containing 0.1% bovine serum albumin (BSA) and 0.1% glucose, pH 7.2.

Cell culture. U937 monocytic cells were obtained from ATCC and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1.0% penicillin-streptomycin solution. The cells were washed twice with HBSS + 0.1% BSA by centrifugation for 5 min at 1000 rpm.

Determination of plasminogen activation and plasmin production. Activation of plasminogen into plasmin was determined by measuring either the plasmin amidolytic activity, or fibrin degradation products. To determine the plasmin amidolytic activity, plasminogen, either soluble (10-20 μg/mL) or immobilized (50 μg/mL) on the surface of fibrin gels or platelets, was incubated for a specified period at 37°C with u-PA (2.5, 5 U/mL) or platelet-bound u-PA (2.5, 5 U/mL) and S-2251 color substrate (0.75 mM). Absorbance was measured at 405 nm using a spectrophotometer. To measure fibrin degradation products, fibrin gels were prepared from ^{125}I-fibrinogen in 24 or 12-well format microplates, and 50–100 μg/mL of plasminogen (250 μL) was incubated with the gels for 15 min at 22 °C. Gels were then washed multiple times with PBS (pH 7.4) to remove any unbound plasminogen. Platelet-bound u-PA or t-PA were prepared by incubating platelets for 30 min at 37 °C with either 10-20 U/mL u-PA or 2 ng/mL t-PA. Platelets were then washed (2-3 times) with Tyrode's buffer containing 0.1 % bovine serum albumin (BSA) and 0.1% glucose. Platelet suspensions (500 μL, $5x10^8$), either u-PA/t-PA-bound or non-u-PA/t-PA bound, were added to fibrin gels with and without immobilized-plasminogen, incubated for a specified period at 37 °C, and radioactivity of fibrin degradation products, released in solution, was measured.

Western Blotting Analysis. Western blotting was performed in order to verify the release of plasminogen activator inhibitor-1 (PAI-1) from fibrin-incorporated platelets but not from adherent platelets. Lysates of platelets and fibrin gel extracts were separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-PAI-1 antibody. As a positive control for PAI-1, 1 µg of PAI-1 protein was run. A single band corresponding to the recombinant PAI-1 was found at approximately 40-50 kDa. A lysate of isolated human platelets ($1x10^9$/ml), prepared by freezing and thawing a suspension of platelets multiple times, was used to determine the total endogenous level of PAI-1.

To measure PAI-1 release from platelets associated with artificial clots, platelets were incorporated into fibrin gels ($5X10^8$/gel, gels prepared as previously described), the gels clotted, and the lysate was collected for measurement of PAI-1. Also, platelets were incorporated into fibrin gels containing U937 monocytes, the gels clotted and PAI-1 release measured. Alternatively, platelets were incubated on top of fibrin gels and were allowed to adhere to the gel surface, the gels clotted, and PAI-1 release measured. For PAI-1 release measurement, 20-µl of each sample lysate was resuspended in 4% SDS sample buffer, boiled for 5 min, and then electrophoresed on a 7.5 % polyacrylimide SDS gel. The gel was then blotted to nitrocellulose using a Tris-based transfer buffer (Tris 25 mM, glycine 190 mM, 20% methanol) and 100v for 1 hr at +4 °C. The membrane was then sequentially probed with primary antibody against PAI-1, horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Bio-Rad). The secondary antibody was detected using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

Formation of DEAE-Sephacel beads containing polymerized fibrin (B-Fb). 1.0 mL of 4.0 mg of fibrinogen or ^{125}I-fibrinogen (^{125}I-Fg) solution ($4x10^6$ cpm) in 10 mM Tris with 1.0 mM Ca^{2+}, pH 7.4, was added to a 1.0 mL DEAE-Sephacel suspension containing 500 mg wet weight beads equilibrated with the same buffer. The mixture was incubated for 5 min at 22 °C and then centrifuged at 500 rpm for 1 min. Beads were washed free of non-bound fibrinogen and resuspended in 4.0 mL Tris-Ca buffer. The dissociation of radioactivity from these beads was negligible. The capacity of DEAE-Sephacel for fibrinogen is 4.0 mg/g of wet weight. Adsorbed fibrinogen was cleaved to fibrin by treating the beads with thrombin (0.5 U/mL) for 1 hr at 22 °C with shaking and then washing with PBS- 1% BSA buffer. To observe adhesion of platelets or U937 cells on the bead's surface, 50 mg of fibrin-coated beads were mixed with platelets ($1x10^{8)}$ or U937 cells ($1x10^7$) in 100 ml of 1% BSA-Hank's buffer and incubated for 30 min at 37 °C. Beads were then washed with 1% BSA-Hank's buffer to removes any non-adherent platelets or U937 cells.

To determine the amount of plasminogen (Pg) that can be bound to DEAE-Sephacel beads, 500 µl of a fibrin-coated bead (B-Fb) suspension (250 mg wet weight) was mixed with 500 µl of Pg) solution (100 µg/mL) in 1% BSA-Hank's buffer. After incubation for 15 min at 22 °C, non-bound Pg was removed by washing with 1% BSA-Hank's buffer. Experiments with ^{125}I-plasminogen showed that 3.0 ±0.4 µg Pg was bound per 100 mg of B-Fb beads.

Fluorescence Confocal Microscopy. Filtered human platelets were labeled with PKH26 using the PKH26 Red Fluorescent Cell Linker Kit according to the

manufacturer's instructions. Briefly, platelets ($1x10^8$/mL) were suspended in 1 mL of Diluent C buffer, mixed with 1 mL of Diluent C buffer with added PKH26 ethanolic dye solution (4 μL), and then incubated for 5 min at 37 °C. Platelets were then washed once with Tyrode's buffer containing 0.1 % bovine serum albumin (BSA) and 0.1% glucose. Aliquots of dye-labeled platelets ($1x10^6$) were either adhered to or incorporated inside fibrin gels. Fibrin gels (50 μL) were prepared on poly-L-Lysine coated microscope slides by mixing 1.5 mg/mL fibrinogen and 0.25 U/mL thrombin. Fibrin gels were allowed to polymerize for 60 min at 22°C. The fibrin gel specimens were then fixed with 2% paraformaldehyde (in TBS) for 30 min at 22 °C, washed 3 times with TBS buffer and further processed for PAI-1 immunocytochemistry. Blocking of gel specimens was done using 1% bovine serum albumin (BSA) in TBS buffer for 60 min at 22 °C, and followed by washing with TBS. Mouse mAb anti-PAI-1 (10 μg/mL) was then added and the specimens incubated for 90 min at 22 °C before being washed 4 times, 15 min each, with TBS. Secondary antibody Alexa Fluor 488, goat anti-mouse (10 μg/mL) was then added and incubated for 90 min at 22 °C, after which samples were washed with TBS 4 times for 15 min each. A mounting medium (Vectashield) was applied to each sample before adding the coverslips. Release of PAI-1 from platelets was visualized using a fluorescence confocal microscope (Leica TSC SP5, Buffalo Grove, IL) at excitation wavelengths of 488 (green: PAI-1) and 633 (red: platelets).

Scanning Electron Microscopy (SEM). Fibrin-bound Sephacel beads were prepared as described above. After fibrin polymerization, beads were washed with Tris-Ca buffer (3 times), fixed with 2% glutaraldehyde in 1x PBS, pH 7.2, for 2 hrs at 22 °C,

then washed through several changes of 1x PBS for 5 min each. Beads were then transferred to poly-D-lysine-coated cover slips, placed in a 12-well format plate, and post-fixed with 1% osmium tetroxide (OsO_4) in 1x PBS (pH 7.4) for 15 min at 22 °C. Following post-fixation, beads were washed for 5 min with Ultrapure® water. Sephacel beads were dehydrated using an ethanol series, transitioned into 100 % acetone, then dried using a Balzers critical point dryer. After mounting onto aluminum stubs, the specimens were sputter coated with palladium-gold under vacuum (Technicks Hummer II) and imaged using a JOEL JSM-6300 scanning electron microscope.

RESULTS

Platelets facilitate the PA-mediated activation of surface-bound plasminogen through an interfacial mechanism. The ability of platelets to assemble the plasminogen activation system on their surface, i.e. to bind plasminogen and its activators urokinase-type (u-PA) or tissue-type (t-PA) activator followed by plasmin generation, has been described in numerous studies (Miles & Plow, 1985; E. F. Plow, Freaney, Plescia, & Miles, 1986; Gao, Morser, McLean, & Shuman, 1990; Loscalzo, Pasche, Ouimet, & Freedman, 1995; Miles, Ginsberg, White, & Plow, 1986; Baeten et al., 2010). However, in these systems both plasminogen and its activator were bound to the same platelet surface.

Extending these results, we show in Fig. 14A that platelets (Plt) with bound u-PA are capable of facilitating the activation of soluble plasminogen (Plt-uPA/Pgsol) and conversely soluble u-PA is able to activate platelet-bound plasminogen (Plt-Pg/uPAsol). In this system, soluble plasminogen and bound plasminogen were activated 45-fold and

25-fold respectively when measured as an increase in plasmin activity detected by the chromogenic color substrate S-2251. Moreover, we found that activation of platelet-bound plasminogen was observed even when plasminogen and u-PA were adsorbed to the surface of a different platelet population (Fig.14A, Plt-uPA/Plt-Pg). In contrast, activation was not seen in this system if neither u-PA or Pg were surface bound (Dissociated uPA,Pg).

In order to further study this interfacial activation, we sought to determine whether fibrin-bound u-PA could activate plasminogen bound to either platelets or fibrin. Indeed, as shown in Fig. 14B, surface-bound u-PA, whether on the surface of platelets (Plt-uPA) or a fibrin gel (Fb-u-PA), is able to activate plasminogen (Pg) bound to a different platelet (Plt-Pg) or fibrin gel (Fb-Pg) surface. In this case, plasminogen activation and plasmin generation was actually measured by release of radioactive fibrin degradation products rather than by an artificial substrate. These data suggest that plasminogen and its activators don't necessarily need to be on the same platelet surface to have an interaction leading to plasminogen activation and subsequent fibrin degradation. Furthermore, interfacial catalysis enables activation of plasminogen bound to the surface of fibrin or to cells by a plasminogen activator, whether u-PA or t-PA, even when they are localized on separate surfaces. Additionally, these data indicate that platelets can activate either platelet- or fibrin-bound plasminogen and, potentially, could use their fibrinolytic ability during adhesion to fibrin clots.

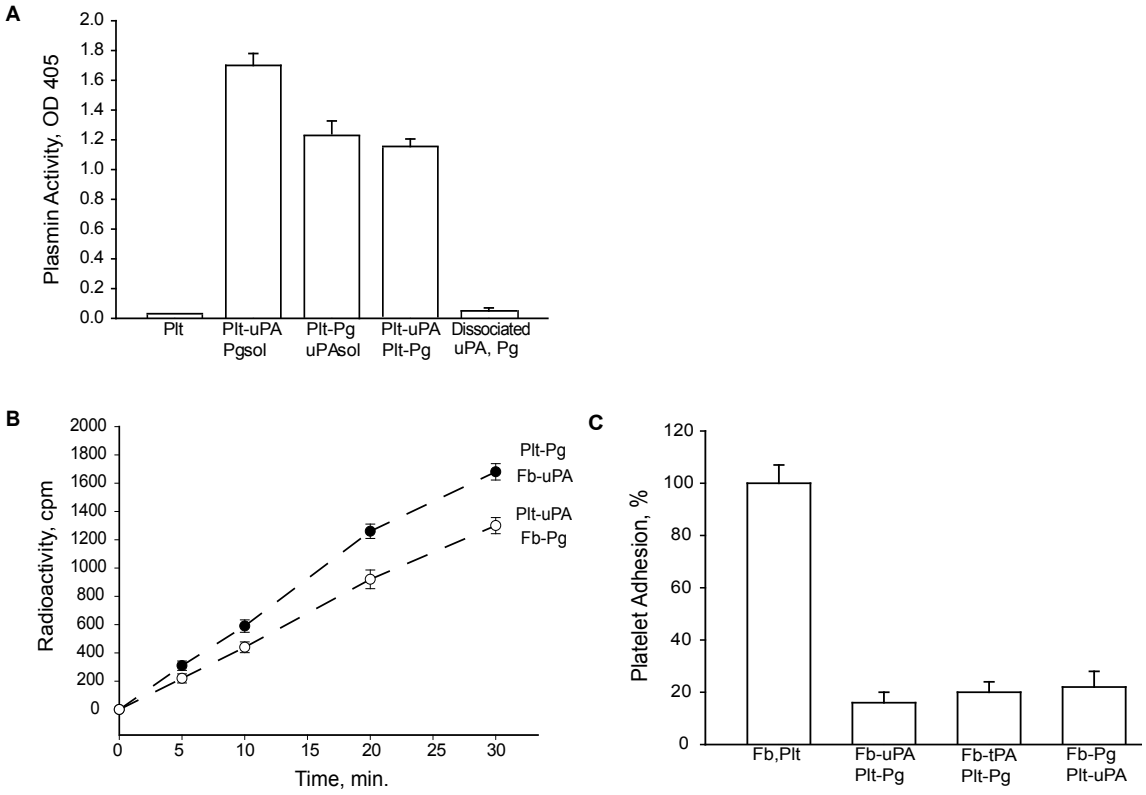


Figure 14. Platelets assemble components of the plasminogen (Pg) activation system on their surface and activate plasminogen through the interfacial mechanism. **(A)** Isolated platelets (Plt) were incubated with either u-PA (20 U/mL) or Pg (50 μg/mL) at 37 °C for 30 min. After washing, platelets ($2x10^8$), with bound u-PA (Plt-uPA) or plasminogen (Plt-Pg) were incubated at 37 °C for 60 min with soluble or platelet-bound Pg (50 μg/mL), or soluble uPA (10 μg/mL), respectively. Platelets without bound u-PA or Pg were used as a control (Plt). To test the effect of uPA or Pg that dissociated from the surface of platelets into solution, platelets were removed by centrifugation and plasmin activity was measured in the supernatant. Plasmin activity was detected using S-2251 and absorbance measured at 405 nm. (**B)** I-125 labeled fibrin gel (1.5×10^5 cpm/gel) was incubated with Pg (50 μg/mL) or with u-PA (20 u/mL) for 15 min at 22 °C. Fibrin gels were washed to remove any non-bound Pg or u-PA. Fibrin-bound Pg (Fb-Pg) or u-PA (Fb-uPA) was incubated with platelet ($2x10^8$)-bound u-PA (Plt-uPA) or Pg (Plt-Pg), respectively, for 5-30 min at 37 °C. Pg activation and plasmin generation was measured by the solubilized radioactivity. (**C)** Platelet-mediated Pg activation measured in the form of platelet adhesion to fibrin gels. Fibrin was incubated with Pg (50 μg/mL), t-PA (20 ng/mL) or u-PA (20 u/mL) for 15 min at 22 °C. The same concentrations of Pg and u-PA were applied to calcein-labeled platelets. Fibrin gels and platelets were washed to remove any non-bound Pg, t-PA or u-PA. Calcein-labeled platelets ($2x10^6$) with bound Pg or u-PA were applied to the fibrin-bound u-PA (Fb-uPA), t-PA (Fb-tPA) or Pg (Fb-Pg) and incubated for 30 min at 37 °C. Pg activation and plasmin generation was measured as a percentage of adherent platelets estimated by the level of fluorescence.

Such fibrinolytic processes on the surface of a fibrin clot could be an effective anti-adhesive mechanism. This anti-adhesive effect was in fact observed (see Fig. 14C). Fluorescent platelets, their surfaces bound with either Pg or u-PA, were allowed to adhere to fibrin gels to which the conjugate member of the system was bound. Subsequently, the effect of these interfacial plasminogen activation systems on platelet adhesion was observed as an 80 to 85% decrease in platelet adhesion regardless of the surface to which each component was bound.

Platelets adherent to adhesive surfaces do not inhibit plasmin activity. Next, we tested whether platelets adherent to different substrates, other than fibrin clots, could affect the u-PA-mediated activation of plasminogen through the release of PAI-1, an inhibitor of plasminogen activators (PAs). The wells of a 24-well format plate were coated with polymerized fibrin gel (Fb gel), fibrinogen (Fg), and fibronectin (Fn), surfaces known to support platelet adhesion and spreading under static and flow conditions (Baumgartner, 1973; S. Falati et al., 2002). Isolated platelets were allowed to adhere to coated wells and uncoated wells as a control at 37 °C (see Methods). After a specified period of time, supernatants containing secreted proteins were assayed for inhibition of plasminogen activation using a soluble u-PA/Plasminogen system and a chromogenic substrate. As shown in Fig. 15A, plasmin activity was not notably affected by the supernatants from adherent platelets, indicating that little or no PA inhibitors such as PAI-1 had been secreted, as compared to the inhibitory activity present in platelet lysates. Moreover, adherent platelets did not release any detectable amount of PA inhibitors even when ADP was added to already adherent cells (not shown). To further

document this lack of PA inhibitor/PAI-1 release, we selected Fb gel- and Fg-adherent platelets for immunofluorescence analysis (Fig. 15B). Platelets were labeled with the PKH26 Red Fluorescent Cell Linker Kit, while PAI-1 was indirectly labeled with Alexa Flour 488, as described in Methods. Visualization using a laser scanning confocal microscope revealed no PAI-1 secretion.

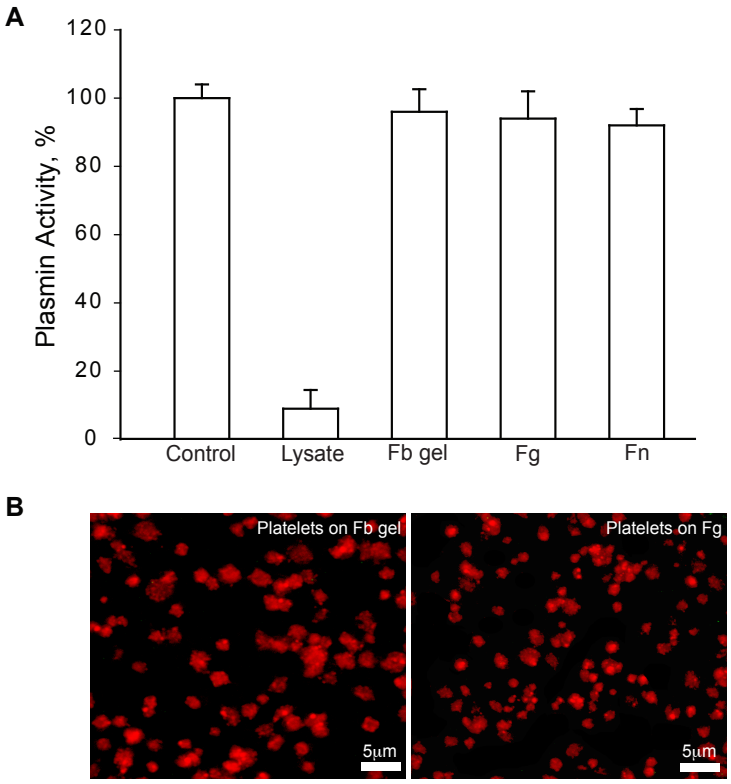


Figure 15. Platelets adherent to different adhesive surfaces do not inhibit plasmin activity. **(A)** Isolated platelets ($5x10^8$ cells/well) were added to non-coated and coated wells with fibrinogen (Fg, 1 mg/ml), fibronectin (Fn, 20 μg/ml), or fibrin (Fb gel). The added platelets were briefly centrifuged (2000 rpm, 1 min) and then incubated for 30 min at 37 °C. Supernatants above the adherent platelets were collected and assayed for its inhibitory effect on plasminogen (Pg) activation. Supernatants were mixed with Pg (20 μg/ml), uPA (5 U/ml), and S-2251 (0.75 μM) and absorbance measured at 405 nm. The control sample reflects samples without the added platelet extract or extract from the equivelant amount of frozen platelets. The lysate represents the extract collected from a frozen/thawed suspension of isolated platelets, which is indicative of the endogenous level of PAI-1. (**B)** Platelets were labeled with the PKH26 Red Fluorescent Cell Linker Kit, while PAI-1 was indirectly labeled with Alexa Flour 488, as described in Methods. Visualization using a laser scanning confocal microscope revealed no PAI-1 secretion.

Plasminogen activation is inhibited by platelets incorporated inside fibrin clots but not by fibrin surface-adherent platelets. Although the ability of platelets to activate plasminogen thereby detaching surface-adherent platelets seems evident, it is not clear how this anti-adhesive mechanism works in the presence of a potent secreted inhibitor such as PAI-1. While platelets house a substantial amount of PAI-1 in their alpha granules, some studies suggest that the vast majority of this PAI-1 is in an inactive form (N. A. Booth, Simpson, Croll, Bennett, & MacGregor, 1988; N. Booth, Croll, & Bennett, 1990; Brogren et al., 2004). On the contrary, other studies have shown that the majority of platelet PAI-1 is able to complex t-PA or u-PA, indicating that PAI-1 is active and able to maintain clot stabilization via inhibition of plasminogen activation (Posan et al., 1998; Fay, Eitzman, Shapiro, Madison, & Ginsburg, 1994; N. A. Booth, Robbie, Croll, & Bennett, 1992). Yet other studies have suggested that PAI-1 acts as the principal factor involved in the resistance of platelet-rich clots to undergo fibrinolysis (Coppinger et al., 2004; Vaughan, 2005; Nieuwenhuizen, 2001). Indeed, as Fig. 16 shows, platelets captured inside fibrin clots suppress the plasminogen activation of both u-PA and t-PA as well as the plasminogen activation ability of U937 monocytic cells. Fig. 16A compares the effect of fibrin gel-incorporated platelets on the u-PA-, t-PA-, and U937 cell-mediated activation of plasminogen. In these experiments, plasminogen (50 μg/mL) was adsorbed to the surface of fibrin gels and the soluble plasminogen remaining washed away so as to specifically focus on interfacial activation of bound plasminogen. In Fig. 16A fibrin gels were prepared either with (black bars) or without (open bars) platelets trapped in their interior. In control gels (Fb gel) activation of bound plasminogen was inhibited 90% by the presence of platelets within the gel. Likewise, addition of t-PA, u-

PA or U937 monocytic cells to the fibrin gel to activate bound plasminogen did not overcome the inhibition by fibrin-incorporated platelets. Thus, when platelets are trapped inside fibrin gels, plasminogen activation, measured in the form of either cleavage of an artificial substrate or release of fibrin degradation products, is almost completely suppressed. This suggests that fibrin-trapped platelets release PAI-1, which inhibits the conversion of plasminogen into plasmin, and subsequently the process of fibrinolysis. Inhibition is observed regardless of whether u-PA or t-PA is included in the fibrin gel or bound to its surface (data not shown).

To expand on these data, we hypothesized that PAI-1 released from fibrin-trapped platelets can also inhibit u-PA that is localized on platelet or U937 cell surfaces when the cell is attached to the clot surface. To test this hypothesis U937 monocytic cells with membrane bound u-PA were either applied to the plasminogen-bound fibrin surface or incorporated inside the fibrin gels in the absence (Fig. 16B, solid circles) or presence (open circles) of fibrin-trapped platelets. Unexpectedly, plasminogen activation by fibrin-surface adherent u-PA-bound-U937 cells was not inhibited by PAI-1 released from the fibrin-incorporated platelets. However, when both u-PA-bound U937 cells and platelets are incorporated inside fibrin gels, plasminogen activation was almost completely inhibited in a platelet density-dependent manner. These results indicate that the anti-fibrinolytic action of PAI-1 released from the fibrin-incorporated platelets is localized within the gel and does not interfere with plasminogen activation occurring at clot's surface in the interface between the fibrin gel and the U937 cell's surface.

To further examine the effect of platelets on the fibrinolytic process, plasminogen activation was studied for its dependence on the amount of platelets either trapped inside

the fibrin gels or adherent to the gel's surface. As shown in Fig. 16C, substantial inhibition in plasminogen activation was observed with only $1x10^7$ platelets/gel, whereas no inhibitory effect was seen with the surface adherent platelets even with $5x10^7$ platelets/gel. These data show that platelets have opposite effects on plasminogen activation depending on their localization within the fibrin clot. Surface-adherent platelets promote plasminogen activation, while fibrin-incorporated platelets significantly inhibit the fibrinolytic process. Furthermore, the data suggest that there is close correlation between the relative PAI-1 content within the thrombus and plasmin generation and thus resistance to fibrinolysis.

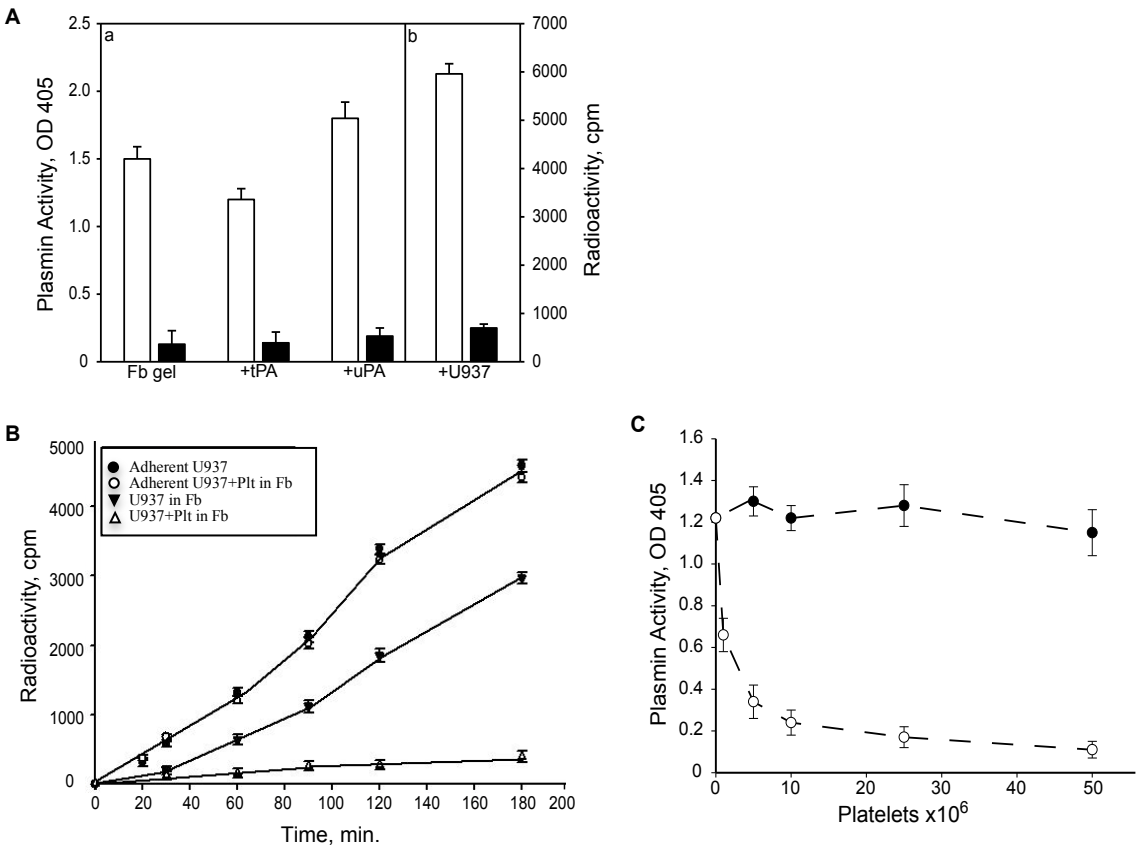


Figure 16. Platelets incorporated inside fibrin gels, in contrast to fibrin surface-adherent platelets, inhibit activation of plasminogen (Pg) by u-PA, t-PA or U937 cells. **(A)** Pg (10 μg/ml) added to fibrin gels made without or with included u-PA, t-PA and platelets ($1x10^9$ /ml). Pg activation was detected using the plasmin color substrate S-2251 (0.75 μM) and absorbance measured at 405 nm. Inhibition of Pg activation in plain fibrin (Fb gel) was measured in the liquid squeezed from the clotted gel using color substrate and uPA as described in Methods. Pg (10 μg/ml) was also added to fibrin gels made with labeled with ^{125}I-Fg ($2x10^5$ cpm/gel) and without or with included platelets (10^9 cell/ml) and U937 ($3x10^6$ cells/ml). Pg activation was measured by solubilized radioactivity. White bars–fibrin gels without included platelets, black bars–fibrin gels with included platelets. (**B)** PAI-1 released from fibrin-incorporated platelets (Plt in Fb) does not affect U937-mediated Pg activation occurring at the surface of the fibrin gel. I-125 labeled fibrin gel ($2x10^5$ cpm/gel) was prepared without or with included platelets ($1x10^8$ cells/gel) and without or with included or surface adherent U937 cells ($1x10^6$ cells/gel). Pg solution (50 μg/ml) was added to the gels for 15 min at 22 °C. U937 cells were added to the gel's surface and allowed to adhere at 37 °C. Solubilized radioactivity was counted over a time interval ranging from 0 to 180 min. (**C)** The anti-fibrinolytic action of PAI-1 is dependent on the amount of platelets incorporated inside fibrin gels. Fibrin gels were made with included uPA (5 μg/ml). Different amount of platelets were either incorporated inside the fibrin gel or applied to its surface and allowed to adhere. Pg (20 μg/ml) was added to the gels and incubated at 37 °C. Plasmin activity was detected using S-2251 and absorbance measured at 405 nm.

Fibrin-incorporated platelets but not surface adherent platelets release substantial amounts of PAI-1. Plasminogen activator inhibitor-1 (PAI-1) is the major, specific, and fast-acting inhibitor of plasminogen activators, both u-PA and t-PA. Since, PAI-1 is released from the alpha granules of activated platelets during blood clot formation, we analyzed and visualized its localization within fibrin gels by western blotting and fluorescence confocal microscopy. Platelets were either incorporated inside the fibrin gels or allowed to adhere onto the gel's surface. In selected gels, U937 cells were included inside the fibrin gel with platelets.

Western blotting (Fig. 17A) shows that, as expected, release of PAI-1 from platelets is dependent on their location within the fibrin clot. When inside the fibrin clot, platelets released substantial amounts of the inhibitor into the clot regardless of whether U937 cells were present (lane 4) or not (lane 3). However, when platelets were adherent to the fibrin surface virtually no PAI-1 secretion was detected (lane 5). Confocal microscopy (Fig. 17B) further confirmed the abundant PAI-1 release (in green, left panel) from fibrin-incorporated platelets (red, left panel) but not from surface adherent platelets (right panel). This supports the hypothesis that platelets have a dual role in fibrin clot formation; release of PAI-1 inside the fibrin clot to help protect it from early dissolution and activation of the fibrinolytic process at the surface of the clot to prevent excessive cell accumulation and further thrombus growth.

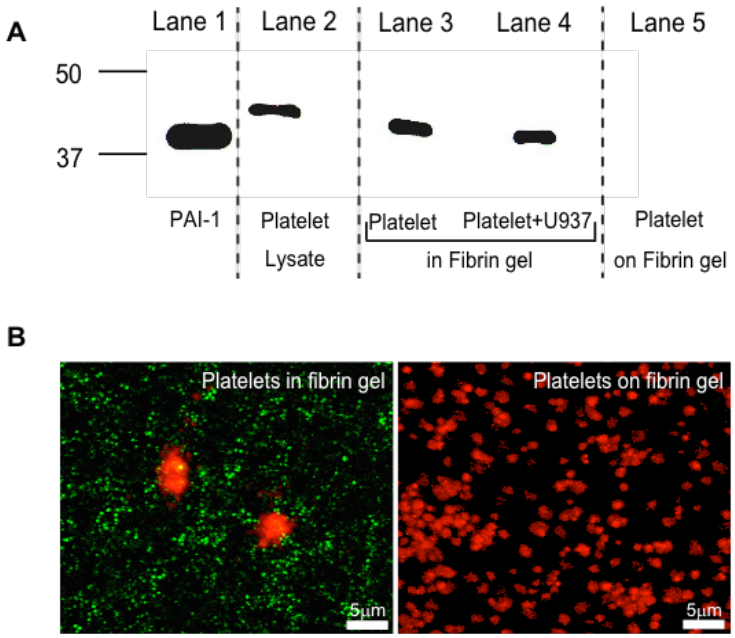


Figure 17. Release of PAI-1 from platelets is dependent on their distribution among the fibrin gel. **(A)** Lysates of gel extracts were electrophoresed, transferred to nitrocellulose and probed with anti-PAI-1. As a positive control for PAI-1, 1 µg of PAI-1 protein was run. Lane 1, an approximately 40-kDa band corresponding to the recombinant PAI-1. Lane 2, the endogenous level of PAI-1 obtained from platelet lysates. Lane 3 and lane 4, bands corresponding to lysates obtained from clotted platelet-incorporated fibrin gels without and with included U937 cells, respectively. Lane 5, band corresponding to lysates collected from fibrin surface-adherent platelets. In lanes 2-5 a consistent amount of protein was electrophoresed. **(B)** Laser scanning confocal microscopy confirms that a substantial amount of PAI-1 is released from platelet-incorporated inside fibrin gels (left panel) but not from fibrin surface-adherent platelets (right panel). Platelets are shown in red and PAI-1 is shown in green.

α2-antiplasmin has no effect on plasmin activity occurring at the interface between the fibrin clot and the cell surface. We sought to investigate whether α2-antiplasmin, a potent direct and rapid inhibitor of plasmin itself (Rijken & Lijnen, 2009; Cesarman-Maus & Hajjar, 2005; Wiman & Collen, 1978), typically available in plasma at high concentration, could interfere with the interfacial plasminogen-activation system of transiently clot-adherent cells. As in the case of PAI-1, α2-antiplasmin could potentially play a key role in the proteolytic anti-adhesive process, despite the fact that plasmin bound to fibrin is less sensitive to the inhibitor than in solution (Hall, Humphries, & Gonias, 1991; M. W. Mosesson, 2005). To detect α2-antiplasmin inhibition, however, we needed to overcome the technical difficulty of measuring plasmin activity at the earliest moment of cell attachment to the fibrin clot surface.

We used DEAE-Sephacel beads as a matrix for fibrin polymerization in order to vastly increase the surface area available for interfacial plasmin activation. 100 mg of wet weight Sephacel has a surface area of about 100 m^2, which can accommodate $1x10^8$ cells as large as U937 monocytic cells. Beads were saturated with ^{125}I-Fibrinogen then treated with thrombin to produce ^{125}I-Fibrin-coated beads. The fibrin coating both within and on the surface of these beads was easily visualized by scanning electron microscopy (Fig. 18B) and platelets readily adhered to the fibrin-coated bead surface (Fig. 18C). Likewise, adherence of much larger U937 monocytic cells to these coated beads was readily visualized by phase contrast microscopy (Fig. 18D). Thus, these micrographs show that DEAE Sephacel beads not only support fibrin polymerization but also adhesion of cells including platelets and U937 monocytes. These ^{125}I-Fibrin coated beads exhibited an extremely low background release of radioactivity (data not shown) allowing us to detect

U937-stimulated fibrinolysis and the effect of α2-antiplasmin on plasmin activity (Fig. 18E). During the first 5 min. of U937 cell contact with fibrin-coated bead to which plasminogen had been pre-bound, fibrin was degraded at a constant rate of about 1300 cpm/min due to plasmin activation at the fibrin-cell interface (solid triangles). Addition of α2-antiplasmin did not inhibit release of fibrin degradation products in this system (open triangles). In contrast, in a non-interfacial system in the absence of cells, we could easily detect the ability of soluble plasmin to release fibrin degradation products from these beads as well as the rapid inhibition of this process by soluble α2-antiplasmin (solid circles).

Taken together these data indicate that neither PAI-1 released from platelets nor α2-antiplasmin present in plasma are capable of interfering with the plasminogen activation reaction that takes place at the interface between the surface of a fibrin clot and an attached cell. Therefore these inhibitors do not prevent cell detachment resulting from this interfacial proteolytic mechanism.

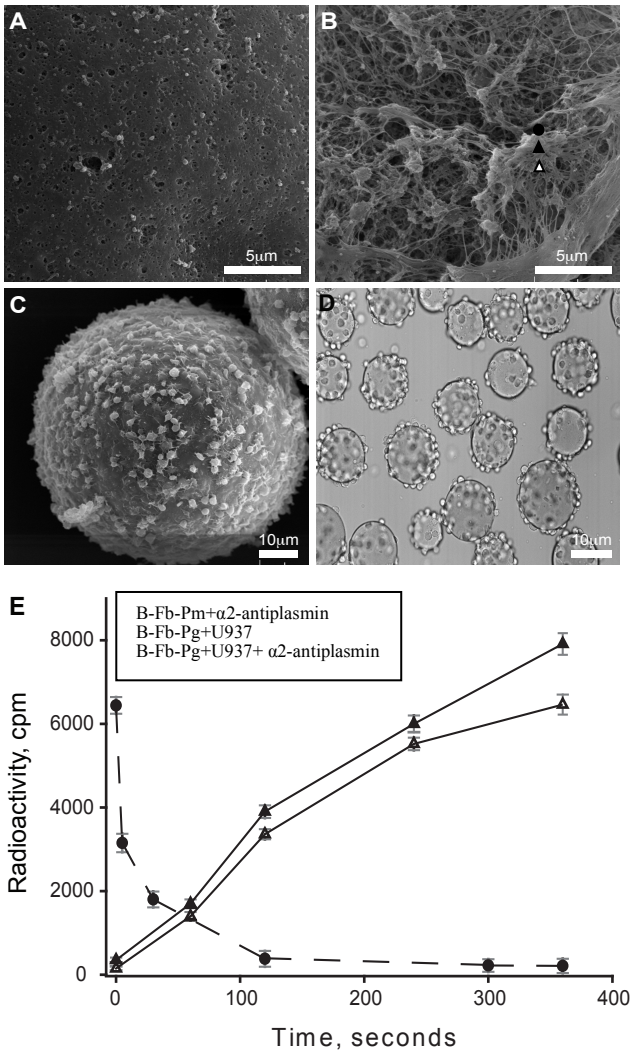


Figure 18. α2-antiplasmin does not affect plasminogen (Pg) activation occurring at the interface between the fibrin and cell surface. **(A) and (B)** SEM of a non-coated and fibrin-coated DAEA-Sephacel beads, respectively. **(C)** Scanning electron micrograph of platelets adherent to the surface of fibrin-coated beads. **(D)** U937 cells adherent to the surface of fibrin-coated beads. **(E)** Effectiveness of α2-antiplasmin against plasmin in a cell-fibrin surface interface and the plasmin exposed to the inhibitor after cell detachment. Pg bound to fibrin-coated beads (B-Fb) was initially activated by incubation with U937 cells. Beads were washed of cells by cold Hank's-BSA (1%) buffer. 100 ml of pre-warmed Hank's (37 °C) with or without α2-antiplasmin (20 μg/ml) was added to 50 mg wet beads then followed by the addition of 1.0 ml cold buffer at a time interval from 5 to 360 sec. Beads were washed with cold Hank's and then incubated for 60 min at 37 °C. Radioactivity released in the solution was counted (black circles). Pre-warmed B-Fb-Pg was mixed with U937 cells ($2x10^7$ cells) with (clear triangles) or without (black triangles) α2-antiplasmin (40 μg/ml). 10 ml aliquots were taken in 1,2,5 and 6 min, diluted to 200 μl, centrifuged at 500 rpm for 1 min, and 50 μl of the supernatant was used to count solubilized radioactivity.

DISCUSSION

The formation of a hemostatic thrombus during blood vessel injury is a highly regulated event, in order to ensure that a blood clot is sufficiently stable to seal the breach but not so overly robust that it causes continuous thrombus growth, and eventually vessel occlusion. In this study we have identified multiple processes that contribute to obtaining this delicate dynamic balance during thrombus formation.

The first objective of thrombus growth and stability is achieved not only by the well-studied process of fibrinogen cleavage and fibrin polymerization (J. W. Weisel & R. I. Litvinov, 2013; Robbie, Booth, Croll, & Bennett, 1993) but also by the fact that the opposing fibrinolytic activity of plasmin, which degrades fibrin, can be held in check by two well-known inhibitors (Groves et al., 1982). PAI-1, which is released from the alpha granules of, activated platelets and α2-antiplasmin, which is present at high concentrations in the plasma. Indeed, in this study we have demonstrated that platelets in the interior of artificial fibrin clots, presumably activated by integrin signaling, release large amounts of PAI-1 as demonstrated by both western blotting (Fig. 17A) and immunolocalization (Fig. 17B). These findings are also supported by previous *in vitro* as well as *in vivo* studies (N. Booth, Croll, & Bennett, 1990; Brogren et al., 2004; Posan et al., 1998; Fay, Eitzman, Shapiro, Madison, & Ginsburg, 1994; N. A. Booth, Robbie, Croll, & Bennett, 1992; Coppinger et al., 2004; Vaughan, 2005; Nieuwenhuizen, 2001). This mechanism likely prevents degradation of the internal platelet-fibrin network that is the basis for thrombus stability needed to plug a vascular breech.

The second objective is to limit enlargement of the thrombus beyond that needed for stabilization. This, by nature, must occur at the surface of the clot, which is covered

with fibrin. Fibrin supports strong integrin-mediated adhesion of both activated and resting platelets *in vitro,* and therefore it would be expected to support accumulation of these cells on the surface of stabilized hemostatic clots *in vivo* and thus continuous thrombus growth. Nevertheless, many *in vivo* studies did not detect any platelet accumulation on the thrombus surface (Kinlough-Rathbone, Packham, & Mustard, 1983; Kamocka et al., 2010; Cooley, 2011; Kerins, Roy, FitzGerald, & Fitzgerald, 1989). These findings implicate the existence of natural processes that operate on the surface of the thrombus, making it non-adhesive to platelets. We have identified two anti-adhesive processes operating on the surface of fibrin clots that prevent excessive accumulation of platelets as well as leukocytes. The binding of fibrinogen or plasminogen to fibrin creates a surface that is mechanically unstable through non-proteolytic and proteolytic mechanisms, respectively. As a result, these plasma proteins form anti-adhesive surfaces that limit integrin-mediated cell adhesion and continued thrombus growth. Therefore, proper regulation of thrombus formation, stability, and dissolution is dependent on an intricate interplay between adhesive and anti-adhesive mechanisms operating on the surface of fibrin clots.

The results of this study clearly indicate the significant physiological function of platelets in maintaining an optimal balance between thrombus stability and thrombus growth. Additionally, our results suggest that the platelet-derived PAI-1 and its role in stabilization of thrombi may be of greater importance than previously thought to be. Our data support the hypothesis that platelets can either activate or inhibit the fibrinolytic process depending on their location within a thrombus. Fibrin-surface adherent platelets promote clot fibrinolysis (Fig. 14) via their ability to assemble components of the

plasminogen activation system on their surface and activate fibrin-bound plasminogen. Subsequently, plasmin-mediated fibrin degradation results in fragmentation and destabilization of the clot surface rendering it incapable of supporting strong integrin-mediated platelet adhesion and further platelet accumulation. This leads to platelet detachment under flow, therefore limiting thrombus growth and possible vessel occlusion. In contrast, platelets incorporated inside a fibrin clot, once activated, release substantial amounts of PAI-1, thus attenuating the platelet fibrinolytic activation potential and thereby protecting the clot from excessive or premature dissolution (Fig. 16C, Fig. 17). However, this anti-fibrinolytic effect of PAI-1 is localized to platelets only within the interior of the clot. Furthermore, our study revealed that PAI-1 released within the clot does not interfere with the platelet fibrinolytic activity and thus the anti-adhesive mechanism occurring at the surface of the clot (Fig. 16A(b), 3B). Likewise, α2-antiplasmin also did not interfere with plasmin fibrinolytic activity occurring at this same cell-clot surface interface (Fig. 18). These findings indicate that achieving optimal thrombus growth and stability is largely dependent on the local balance of proteases and inhibitors around and within the thrombus. Abnormalities in cellular and/or protein components may lead to either excessive fibrinolysis and premature clot dissolution resulting in bleeding, or excessive clot growth and resistance to fibrinolysis resulting in pathological thrombosis (Potter van Loon, Rijken, Brommer, & van der Maas, 1992; Fujii, Hopkins, & Sobel, 1991; Aso, 2007; Dieval, Nguyen, Gross, Delobel, & Kruithof, 1991).

Taken together, this study sheds light on the previously unrecognized dual role of platelets in regulating thrombus formation. The observation that platelets have both

fibrinolytic and anti-fibrinolytic activities that operate in two different regions of the thrombus may provide valuable insights as to how coagulation and fibrinolysis are intimately connected by highly regulated dynamic processes, which are central to attaining hemostatic balance.

CHAPTER 4

DEPOSITION OF FIBRINOGEN ON THE SURFACE OF IN VITRO THROMBI PREVENTS PLATELET ADHESION

ABSTRACT

The initial accumulation of platelets after vessel injury is followed by thrombin-mediated generation of fibrin, which is deposited around the plug. While numerous *in vitro* studies have shown that fibrin is highly adhesive for platelets, the surface of experimental thrombi *in vivo* contains very few platelets suggesting the existence of natural anti-adhesive mechanisms protecting stabilized thrombi from platelet accumulation and continuous thrombus propagation. We previously showed that adsorption of fibrinogen on pure fibrin clots results in the formation of a non-adhesive matrix, highlighting a possible role of this process in surface-mediated control of thrombus growth. However, the deposition of fibrinogen on the surface of blood clots has not been examined. In this study, we investigated the presence of intact fibrinogen on the surface of fibrin-rich thrombi generated from flowing blood and determined whether deposited fibrinogen is non-adhesive for platelets. Stabilized fibrin-rich thrombi were generated using a flow chamber and the time that platelets spend on the surface of thrombi was determined by video recording. The presence of fibrinogen and fibrin on the surface of thrombi was analyzed by confocal microscopy using specific antibodies. Examination of the spatial distribution of two proteins revealed the presence of intact fibrinogen on the surface of stabilized thrombi. By manipulating the surface of thrombi to display either fibrin or intact fibrinogen, we found that platelets adhere to fibrin- but not to fibrinogen-coated

thrombi. These results indicate that the fibrinogen matrix assembled on the outer layer of stabilized *in vitro* thrombi protects them from platelet adhesion.

INTRODUCTION

The formation of a hemostatic thrombus during blood vessel injury is a highly regulated process. It ensures that a blood clot is sufficiently strong to seal the breach and prevent the loss of blood, but is not overly robust to cause vessel occlusion. The response to vascular injury is initiated by adhesion of platelets to the exposed sub-endothelium followed by their aggregation (reviewed in (Z. M. Ruggeri & Mendolicchio, 2007; B. Furie & Furie, 2008)). Formation of the platelet plug is spatially coordinated with the activation of the blood coagulation system leading to thrombin generation and fibrin formation, the latter serving as a scaffold essential for the mechanical stability of the mixed fibrin/platelet thrombus. Depending on the type and severity of injury, a variable amount of fibrin has been detected within and around platelet thrombi in experimental animal (Jorgensen, Rowsell, Hovig, & Mustard, 1967; Sixma & Wester, 1977; Hatton, Ross, Southward, DeReske, & Richardson, 1998; van Ryn et al., 2003). Fibrin formation has also consistently been observed in *ex vivo* models of thrombosis (Weiss, Turitto, & Baumgartner, 1986; Gast, Tschopp, & Baumgartner, 1994; Mailhac et al., 1994; Kirchhofer, Tschopp, Steiner, & Baumgartner, 1995).

Since uncontrolled blood coagulation is potentially dangerous, different anticoagulant mechanisms are activated to limit thrombus growth and localize it to the site of injury (Dahlback, 2005). Even though the formation of fibrin ceases after some time, it is unclear why this fibrin remains non-thrombogenic. Fibrin supports strong

integrin-mediated adhesion of both activated and resting platelets *in vitro* (Hantgan, Hindriks, Taylor, Sixma, & de Groot, 1990; Jen & Lin, 1991; Hantgan et al., 1992; Endenburg, Hantgan, Sixma, de Groot, & Zwaginga, 1993) and therefore, it would be expected to support accumulation of these cells on the surface of stabilized thrombi *in vivo* and thus promotion of continued thrombus propagation. Nevertheless, many studies in experimental animals using traditional staining methods, isotopes, electron microscopy as well as advanced imaging techniques have not detected platelet accumulation on the surface of fibrin (Groves et al., 1982; Kinlough-Rathbone et al., 1983; Kamocka et al., 2010; Cooley, 2011). It has been reported that fibrin-rich thrombi produced in a model of repeated balloon injury in rabbit arteries do not propagate and only become occlusive after a significant reduction in blood flow (Kinlough-Rathbone et al., 1983; Kamocka et al., 2010; Cooley, 2011; Yamashita et al., 2004). Moreover, clinical findings indicate that non-occlusive fibrin-containing coronary thrombi are frequently detected during autopsies of noncardiac death and also present in a large number of subjects with evidence of silent plaque ruptures (reviewed in (Libby, 2008; Arbab-Zadeh, Nakano, Virmani, & Fuster, 2012; Srikanth & Ambrose, 2012; Falk, Nakano, Bentzon, Finn, & Virmani, 2013)). These observations suggest that non-occlusive thrombi are frequently formed and then followed by healing. While these various findings implicate the existence of processes that prevent the accumulation of platelets on the surface of fibrin formed around thrombi, the underlying mechanisms remain poorly understood.

In recent reports using purified proteins and isolated cells we showed that adsorption of fibrinogen on various surfaces, including fibrin clots, results in a dramatic loss of platelet and leukocyte adhesion (V. K. Lishko et al., 2007). The underlying mechanism of

this process involves the adsorption of intact fibrinogen in a thin superficial layer of fibrin clots (V. K. Lishko et al., 2012) and its self-assembly leading to the formation of a nanoscale (~10 nm thick) multilayer matrix (I. S. Yermolenko et al., 2010; Jirouskova, Jaiswal, & Coller, 2007). The fibrinogen matrix is extensible, which makes it incapable of transducing strong mechanical forces via cellular integrins, resulting in weak intracellular signaling and infirm cell adhesion. (I. S. Yermolenko et al., 2010; Jirouskova, Jaiswal, & Coller, 2007). Consequently, the inability of platelets to adhere firmly and consolidate their grip on the extensible fibrinogen matrix leads to their detachment under flow. This interpretation is consistent with other studies that showed that fibrinogen deposited at high density reduces signaling in platelets (Hui, Haber, & Matsueda, 1983). Since thrombi in the circulation are continuously exposed to high (2-3 mg/mL) concentrations of fibrinogen, we hypothesize that the non-adhesive fibrinogen matrix assembles on the surface of fibrin developed around thrombi thereby preventing platelet adhesion and accumulation.

This study was undertaken to determine whether the surface of stabilized thrombi exposed to blood is in fact covered with intact fibrinogen and whether deposited fibrinogen has anti-adhesive properties. Given the nanoscale nature of the fibrinogen multilayer, which would make the observation and manipulation of this structure *in vivo* challenging, we utilized a flow chamber to generate fibrin-rich thrombi that would mimic hemostatic clots formed under flow. Using specific monoclonal antibodies capable of discriminating between intact fibrinogen and fibrin, we analyzed the spatial distribution of fibrinogen and its association with fibrin. We also manipulated the surface of thrombi

to display either intact fibrinogen or fibrin to demonstrate that fibrinogen prevents adhesion of platelets.

MATERIALS AND METHODS

Materials. Human fibrinogen and thrombin were obtained from Enzyme Research Laboratories (South Bend, IN). Monoclonal antibody (mAb) 59D8 which recognizes the N-terminal end of the β-chain of human fibrin (Runge, Bode, Matsueda, & Haber, 1987; Mizuno et al., 2008) was obtained from Dr. H. Weiler, Blood Center of Wisconsin. MAb FPA directed against the fibrinopeptide A of human fibrinogen was a gift from Dr. J. Shainoff, the Cleveland Clinic. Polyclonal antibody against β_3 integrin subunit was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The following secondary antibodies were obtained from Invitrogen (Life Technologies, Grand Island, NY): goat anti-mouse antibodies conjugated to Alexa 488, 633 or 647, and donkey anti-rabbit antibodies conjugated to Alexa 568. Alexa 488-labeled fibrinogen was from Invitrogen (Life Technologies, Grand Island, NY). MAb 7E3-targeting protein was a gift from Dr. B. Coller. Thrombin inhibitors argatroban monohydrate and hirudin were from Sigma (St. Louis, MO) and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) was from Haematologic Technologies Inc. (Essex Junction, VT). The chromogenic thrombin substrate S-2238 was purchased from Sigma.

Parallel plate flow chamber. Glass coverslips (22x22 mm) were plasma cleaned and incubated with a solution of rat-tail collagen type I (BD Biosciences, Bedford, MA)

at 3.5 mg/mL. Glass coverslips were placed in a custom parallel plate flow chamber (flow path of 4 mm width, 16 mm length and 0.13 mm height), and the chamber was assembled for perfusion studies. Blood for perfusions was treated with 6 μM argatroban. This concentration of argatroban does not inhibit thrombin completely and allows for sufficient fibrin formation (Hattori et al., 1978), which is necessary to generate fibrin-rich thrombi. Complete inhibition of thrombin would result only in a platelet plug without any deposited fibrin. Blood was then drawn for 15 min through the chamber by a Harvard Apparatus pump producing estimated shear rates of 300, 1500 and 3000s^{-1}. In selected experiments, 50 or 100 μM PPACK, 200 μM argatroban or 2.5 μM hirudin were perfused over the formed thrombi at 300-1500 s^{-1} for 15 min to inactivate thrombin generated on the surface of thrombi. In other selected experiments, a flow chamber containing thrombi generated at 3000 s^{-1} was mounted on the stage of a Leica inverted microsocope (DM IL, Buffalo Grove, IL), and platelets (1 x10^7/mL) in Tyrode buffer (137 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 0.2 mM Na_2HPO_4, 12 mM $NaHCO_3$, 5.5 mM glucose, 0.1% BSA, pH 7.2) were perfused at 1500 s^{-1}. Images were obtained every second, and the contact time of platelets with selected thrombi was determined.

Preparation of thrombi covered with fibrinogen or fibrin. Three types of thrombi displaying on their surface either intact fibrinogen or fibrin were prepared as follows: (1) thrombi generated for 15 min at 3000 s^{-1} were perfused with Tyrode buffer containing 100 μM PPACK for 15 min at 1500 s^{-1} followed by 15 min resting in the perfusion solution ("fibrinogen" surface); (2) thrombi generated for 15 min at 3000 s^{-1} were perfused with Tyrode buffer supplemented with 0.1 U/mL thrombin for 15 min followed by 15 min of rest in the same solution ("fibrin" surface); and (3) thrombi generated as in the second condition to obtain fibrin were incubated with 100 mM PPACK for 15 min to inhibit thrombin ("fibrin+PPACK" surface). Subsequently, calcein-labeled platelets ($1x10^7$) in Tyrode buffer, containing 5.5 mM glucose and 0.1% BSA, pH 7.2, were perfused over thrombi at a shear rate of 1500 s^{-1} for 15 min.

Fibrinogen and fibrin spatial distribution in/around thrombi using fluorescence confocal microscopy. Thrombi generated on the coverslips were washed with phosphate buffered saline (PBS) or Tyrode buffer and then fixed in 4 % formaldehyde for 15 min at 22 °C. Samples were washed with PBS and incubated with 0.3 % Triton X-100 for 2.5 min, then rinsed and incubated with 3% BSA for 60 min. Samples were washed and then incubated with solutions containing various combinations of anti-fibrin (20 μg/mL), anti-β_3 (4 μg/mL) and anti-fibrinopeptide A (20 μg/mL) antibodies for 1.5 h at 37 °C. After washing, samples were incubated with appropriate secondary antibodies for 1.5 h at 37 °C, washed and mounted on glass slides, and viewed using a confocal microscope (Leica TSC SP5, Buffalo Grove, IL). When using different experimental conditions, consistent exposure and laser intensity setting were used. Care

was taken to ensure that images were collected with the maximum dynamic range for 8-bit images (0-255 greyscales). Greyscale images were pseudocolored using Leica AS software and exported as maximum protection TIFF files. Where applicable, brightness and contrast were adjusted linearly and equally for the different channels using ImageJ to aid in visualization of printed images. In experiments where signal intensity was compared (i.e. signal *vs*. no signal), voltage corresponding to the photomultiplier tube gain remained the same between samples. Control experiments were performed with secondary antibodies only. Optical sections of images were produced using the z-stack function. Vertical cross-sections of thrombi were generated using ImageJ software and analyzed for the distribution of fibrinogen and fibrin. The three-dimensional images were constructed using ImageJ.

Characterization of the fibrinogen and fibrin-specific mAbs. To verify the specificity of anti-fibrinogen (FPA) and anti-fibrin (59D8) mAbs used in this study, the microtiter wells of an Immulon 4B plate were coated with different concentrations of fibrinogen overnight at 4 °C. Selected wells were incubated with 1 U/mL thrombin to convert fibrinogen into fibrin. In addition, selected experiments were performed using fibrin-monomer prepared by dissolving preformed fibrin clots in 3 M urea and directly coating fibrin-monomer onto the wells. The wells were blocked with 1% BSA and incubated with 2 μg/mL of 59D8 or FPA mAbs for 1 h at 37 °C. Following washing, the plates were incubated with goat anti-mouse secondary antibody conjugated to alkaline phosphatase for 1 h at 37 °C. The binding of mAbs was determined by adding the substrate p-nitrophenyl phosphate and measuring the absorbance at 405 nm.

Assessment of thrombin activity associated with thrombi. To determine the thrombin activity associated with surface of thrombi, the thrombi were generated in the flow chamber at 3000 s^{-1}, and then Tyrode buffer alone or supplemented with 100 μM PPACK was perfused through the chamber at a shear rate 300 s^{-1} for 15 min. The chamber was disassembled, washed with PBS, and the coverslip was placed in a 35 mm Petri dish containing 2 mL of 25 μM S-2238. The dishes were incubated at 37 °C and absorbance at 405 nm was determined at 10-30 min.

RESULTS

Thrombus formation and fibrin deposition on the surface of thrombi at different shear rates. To examine whether fibrinogen accumulates on the surface of fibrin covering the surface of stabilized thrombi, we initially tested the conditions that produce the largest amount of fibrin. Blood was treated with 6 μM argatroban to allow partial blood anticoagulation (i.e. incomplete thrombin inhibition) and perfused over collagen-coated coverslips at 300, 1500 and 3000 s^{-1} shear rates. These shear rates mimic blood flow conditions found in veins, arteries and small or stenosed arteries, respectively. After 15 min, thrombi were fixed and incubated with antibodies against fibrin and the platelet β_3 integrin subunit. The mAb 59D8 was utilized for labeling based on its ability to selectively interact with fibrin, but not fibrinogen (Fig. 19A). Small platelet thrombi formed at 300 s^{-1} had an irregular shape with little fibrin deposited mainly at their periphery (Fig. 20A). The thrombi formed at high shear rates (1500 s^{-1} and 3000 s^{-1}) were elongated with a distinct thrombus head and tail and contained a core consisting of aggregated platelets (Fig. 20B and Fig. 20C). The morphometric analyses indicated that a

significant portion of thrombi (~80%) generated at 3000 s^{-1} had a width to length ratio in a range of 2-4 (Fig. 21). Fibrin was deposited not only around thrombi but also many thrombi were often connected by fibrin strands. Vertical cross-sections, z-stacks, and three-dimensional reconstruction showed the presence of fibrin deposited on the surface of thrombi generated at 1500 and 3000 s^{-1} (Fig. 20B and 20C). However, variability in the coverage of fibrin was noted, as the uppermost surface of platelet thrombi was not always fully shielded by fibrin. Representative fields containing thrombi and a summary of experiments performed at 1500 s^{-1} and 3000 s^{-1} showing variability of the surface appearance are presented in Fig. 22. The inconsistency in thrombus surface coverage by fibrin may reflect an inter-individual variability in platelet responses to collagen, heterogeneity in collagen coating or other reasons. Despite these variations, thrombi generated at 1500 s^{-1} and 3000 s^{-1} showed extensive deposition of fibrin. Since thrombi formed at 3000 s^{-1} had more fibrin than those at 1500 s^{-1}, the shear rate of 3000 s^{-1} was selected to generate thrombi for subsequent analyses.

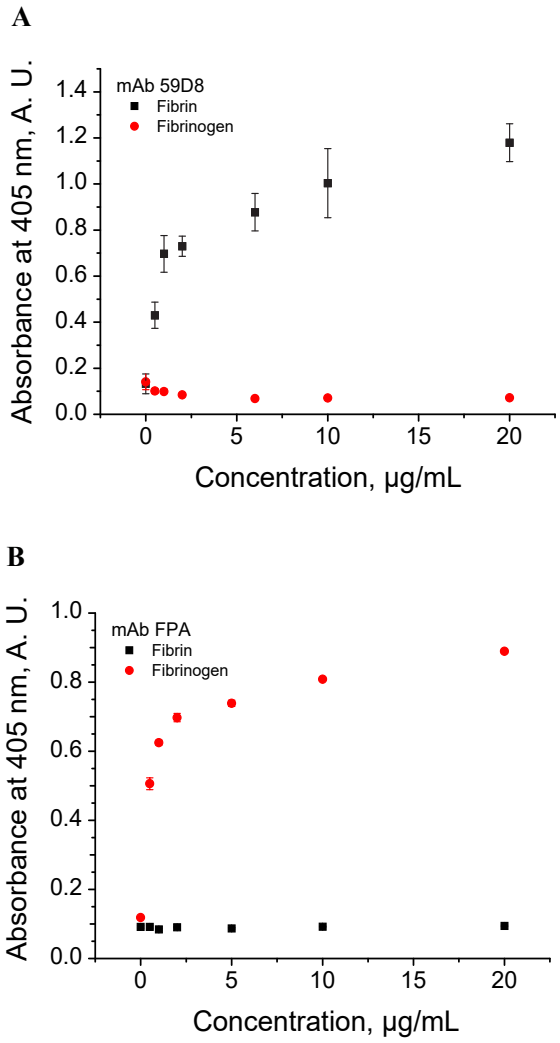


Figure 19. Characterization of antibodies specific for fibrinogen or fibrin. **(A)** mAb 59D8, **(B)** mAb FPA. The data shown are means +/- SD from 3 experiments with triplicate measurements at each experimental data point.

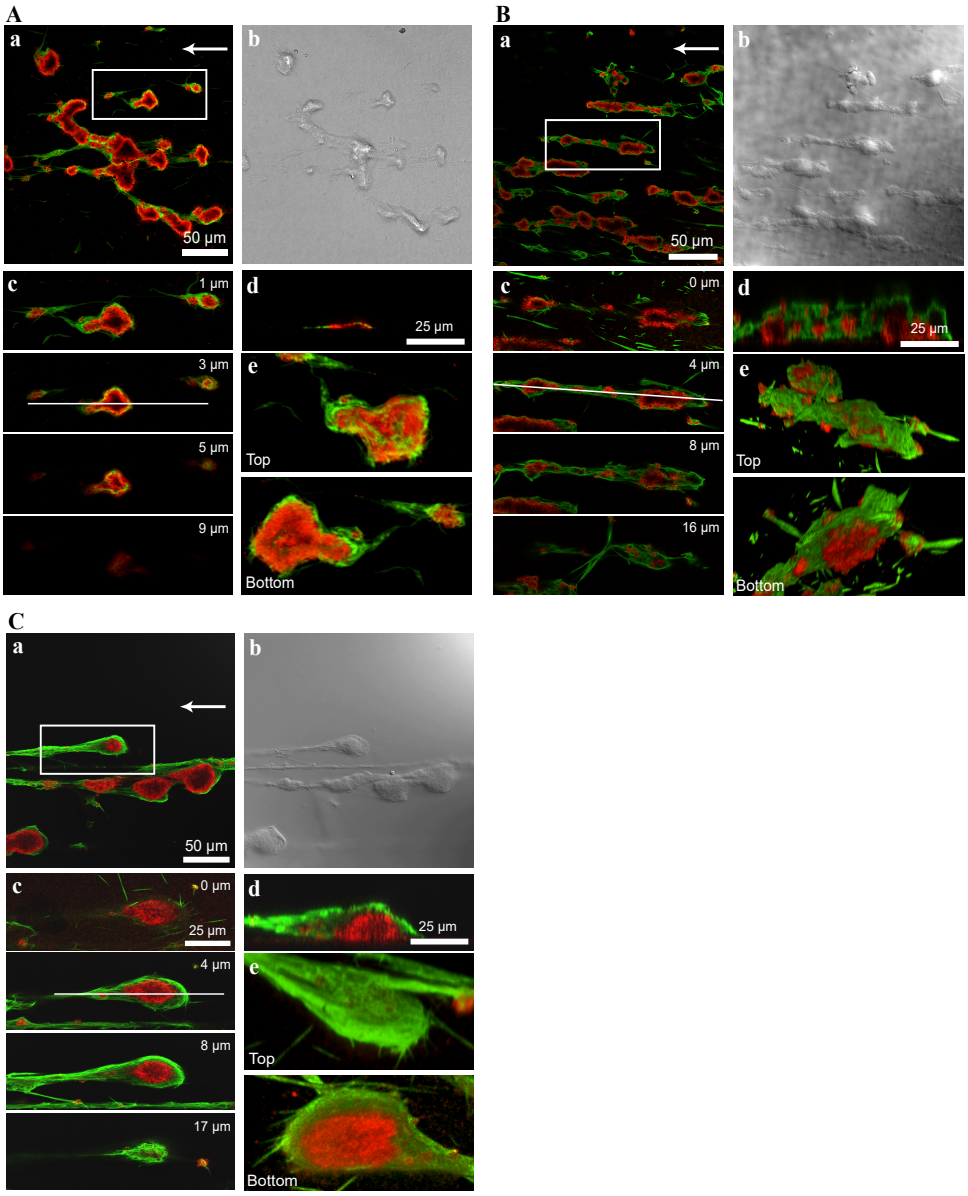
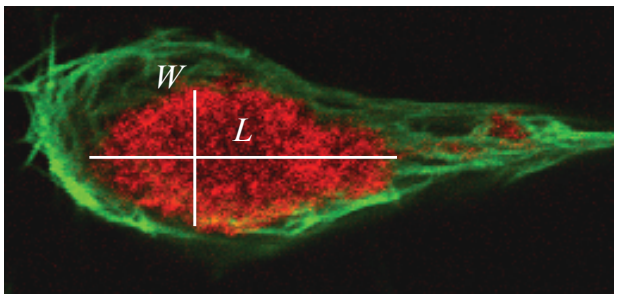


Figure 20. Analysis of fibrin distribution in thrombi formed at a shear rate of 300 s^{-1}, 1500 s^{-1} and 3000 s^{-1}. Whole blood treated with 6 µM argatroban and perfused through the flow chamber containing a collagen-coated coverslip for 15 min at a shear rate of **(A)** 300 s^{-1} **(B)** 1500s^{-1} and **(C)** 3000 s^{-1}. The generated thrombi were stained for fibrin *(green)* using mAb 59D8 and for platelet β_3 integrin *(red)* using polyclonal anti-β_3 antibody. **a,** a representative confocal image of several thrombi. **b,** a DIC image of the field shown in a. **c**, series of cross-sections (z-stacks) of one of the thrombi shown in a (box) taken at 1, 2,5, and 9 µm **(A)**, 0, 4, 8, and 16 µm **(B)**, or 0, 4, 8,and 17 µm **(C)** from the bottom of the chamber. **d**, vertical cross-section of the thrombus shown in c taken at the position shown by a horizontal white line. **e**, three dimensional reconstructions of the thrombus shown in a-c (top and bottom views).

A



B

Ratios:

H/W	W/L	H/L
0.9±0.1	0.5±0.2	0.5±0.1

C

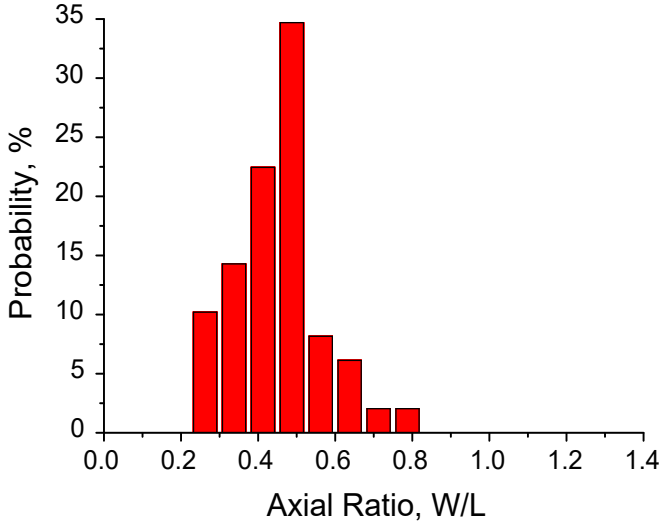
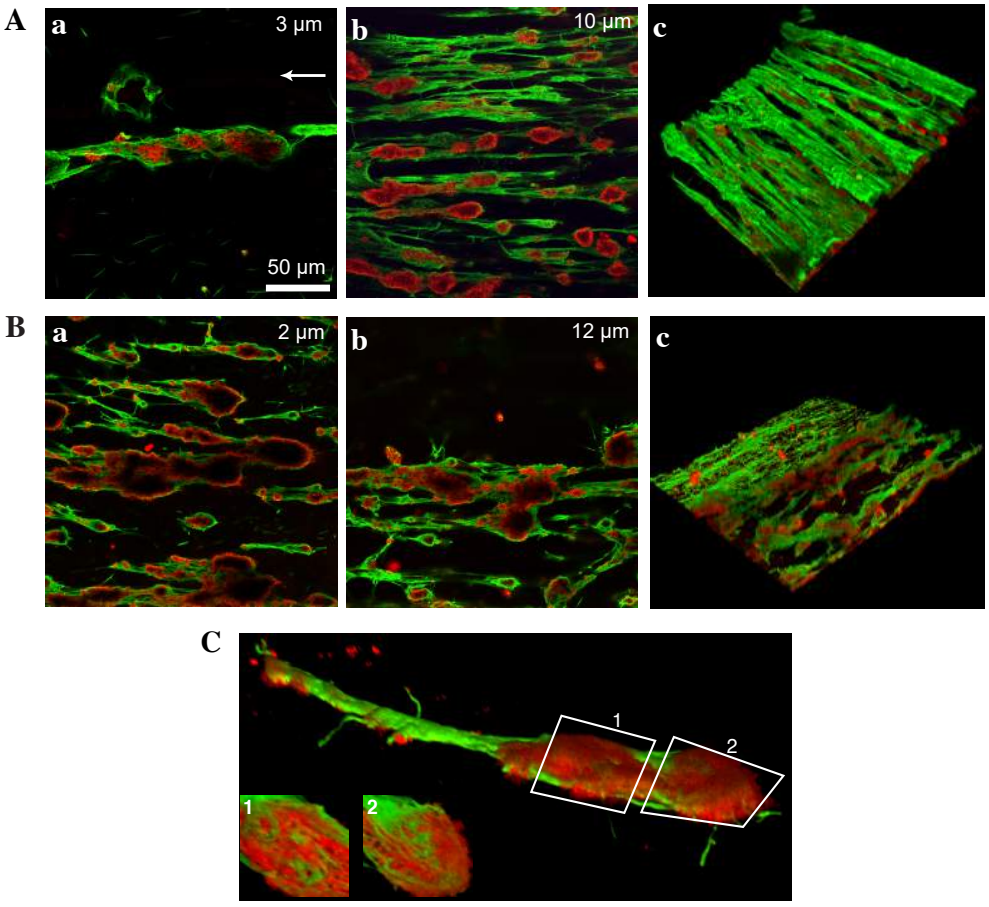


Figure 21. Morphological analyses of thrombi prepared at 3000 s^{-1}. Thrombi generated at 3000 s^{-1}, as described in the Methods section, were labeled for fibrin (green) with anti-fibrin mAb 59D8 and for platelets (red) with anti-β3 antibodies, and their size was determined by measuring the length (L), width (W) and height (H) of the platelet aggregate (red). The length was measured in the direction of the flow, the width was measured in the direction perpendicular to the flow and height was measured as the maximal height of the thrombus. (**A**) Representative thrombus. **(B)** Mean ratios ± SD for L/W, H/W and H/L. The data are for 50 thrombi. **(C)** A histogram of the distribution of the axial ratios (W/L) in the population of analyzed thrombi.



D

	Number of experiments			
Shear Rate s^{-1}	Total	Fibrin cap and fibrin fibers[a]	Fibrin and cells[b]	Predominantly cells[c]
3000	95	43	20	32
1500	10	1	9	0
300	10	5	2	3

a) Full coverage with fibrin and fibrin fibers connecting individual thrombi.

b) Partial coverage of thrombi with fibrin.

c) Thrombi consisting of cells with a minimal amount of fibrin fibers.

Figure 22. Statistical analyses of thrombi generated at different shear rates. Thrombi generated at a shear rate of **(A,C)** 3000 s^{-1} and **(B)** 1500 s^{-1}. Images are the cross-sections of thrombi at different heights from the collagen surface **(a,b)**. **c**, 3D reconstructions of thrombi shown in **b**. **(C)** Representative 3D reconstruction of a selected thrombus showing the distribution of fibrin (green) and platelets (red) and an incomplete fibrin cap. Enlarged images of the boxed areas (1 and 2) show partial staining for fibrin, which is indicative of an incomplete fibrin cap. **(D)** Summary of all blood perfusion experiments.

Adhesion of platelets to the surface of fibrin-rich thrombi under flow. As shown in Fig. 20, there was no platelet-associated immunostaining on the surface of thrombi above the fibrin layer. Moreover, platelets were not detected on the fibrin fibers connecting thrombi (Fig. 20). To further confirm that platelets do not adhere to the surface of thrombi, we performed video recording of flowing platelets. Thrombi were generated by perfusing argatroban-treated whole blood at 3000 s^{-1} followed by washing with platelet-poor plasma to remove blood cells. Platelets in Tyrode buffer were then perfused over the formed thrombi. Live video microscopic images were captured with a frame interval of one second. A representative snap shot of a thrombus with platelets in contact with its surface is shown in Fig. 23A.

The distribution of the average interaction times between platelets and the surface of thrombi is shown in Fig. 23B. The majority of platelets (>70%) do not contact the surface of thrombi longer than 3-4 s, although approximately 10% remain on the surface for more than 20 s (20-100 s). While interacting with the surface of thrombi, these latter platelets remained in motion rolling over the surface with short stops before complete detachment. Since no statistically significant pattern regarding this motion within the interaction time was found, we limited ourselves to the average displacement speed, which was calculated as the distance that a platelet travels on the surface between consecutive frames (Fig. 23C). The analyses showed that the majority of rolling platelets moved with a speed of 2-3 μm/sec. These results indicate that platelets only transiently adhere to the surface of a thrombus, suggesting that it is largely nonreactive for platelets.

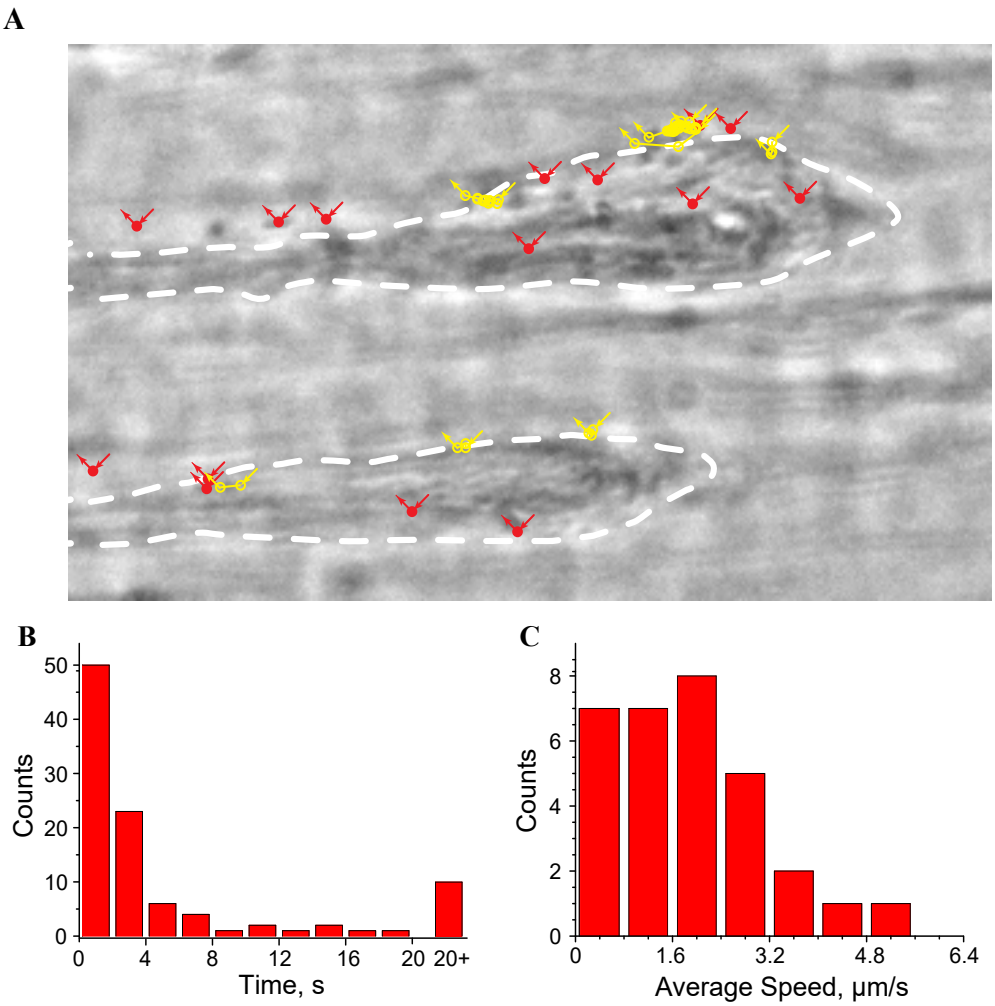


Figure 23. Tracking platelet contacts with the surface of thrombi. Thrombi generated at 3000 s^{-1} were washed with platelet poor plasma (PPP) for 15 min, and then platelets in Tyrode buffer were perfused over the preformed thrombi for 15 min. **(A)** Live video microscopic images of platelets in contact with the thrombus surface were captured with a frame interval of 1 sec. The platelets that stayed on the surface less than 2 s are marked in solid red. The platelets that interacted with the surface of thrombi for 2-30 s are marked in yellow. All platelet positions are marked with circles and displacements are shown as lines. The contours of two thrombi are outlined. **(B)** Distribution of times each platelet spends on the surface of thrombus. The data shown are from 100 platelets. **(C)** Distribution of the average rolling speed of each platelet while on the surface of thrombus expressed as µm/s. n=3 experiments.

Fibrinogen distribution on the fibrin surface of thrombi. To examine the presence of intact fibrinogen on the surface of the fibrin cap, the generated thrombi were washed with Tyrode buffer to remove blood cells and then incubated with fibrinogen-specific mAb FPA, which selectively binds intact fibrinogen but not fibrin (Fig. 19B). As shown in Fig. 24A, the presence of intact fibrinogen on the surface of thrombi was observed. However, fibrinogen was not detected as a uniform coat in association with fibrin but rather appeared in a punctate manner. One reason for this uneven distribution is the presence of fibrin-associated thrombin, which continued to convert fibrinogen into fibrin while the coverslips were being processed for immunostaining. As expected, we detected the presence of thrombin activity associated with thrombi using a chromogenic thrombin substrate S-2238 (Fig. 25).

To investigate the possibility that fibrin-bound thrombin decreased the amount of intact fibrinogen, the generated thrombi were washed with Tyrode buffer supplemented with 50-100 μM PPACK. PPACK-treated thrombi were incubated with mAb FPA and found to exhibit a stronger and more uniform staining for fibrinogen (Fig. 24B). To further explore the capacity of fibrinogen to adsorb on fibrin, PPACK-treated thrombi were perfused with platelet-rich plasma containing Alexa 488-labeled fibrinogen. As shown in Fig. 24C, thrombi accumulated Alexa 488-labeled fibrinogen, which predominantly colocalized with fibrin. Finally, nonlabeled fibrinogen (2.7 mg/mL) was perfused over PPACK-treated thrombi and found to adsorb on the surface of thrombi as disclosed by staining with mAb FPA (Fig. 24D, a-d). Exogenously added unlabeled (Fig. 24D) and Alexa 488-labeled fibrinogen (Fig. 24C) decorated the thrombus head and tail and also coated fibrin fibers connecting thrombi. Since, in each case, fibrinogen was

added after thrombi formation, the deposition of fibrinogen evidently occurs on the surface of thrombus-associated fibrin.

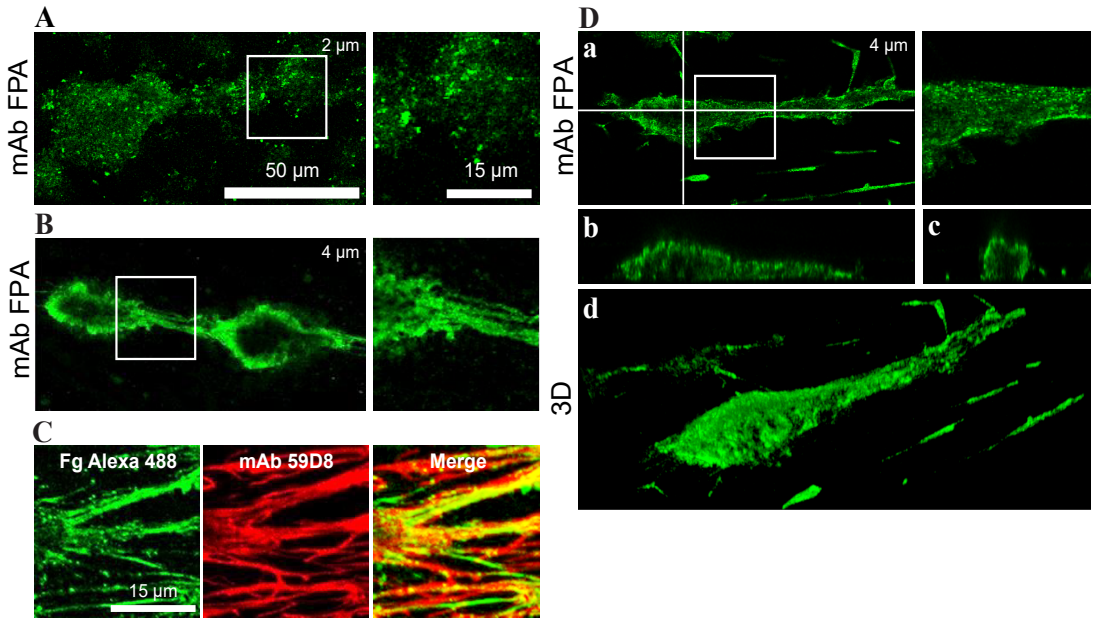


Figure 24. Deposition of fibrinogen on the surface of thrombi. Thrombi generated at 3000 s^{-1} and washed with Tyrode buffer for 15 min. **(A)** Thrombi incubated with anti-fibrinogen mAb FPA followed by Alexa 488-secondary antibody *(green)*. The right panel shows an enlarged area (box) from the left panel. Image shown is taken at 2 µm from the bottom of the chamber. n=3 experiments. **(B)** Thrombi were washed to remove blood cells followed by 15 min perfusion with PBS+50 µM PPACK at 1500 s^{-1}. Thrombi were incubated with mAb FPA *(green)*. The right panel shows an enlarged area (box) from the left panel. The image shown is taken at 4 µm from the bottom of the chamber. n= 3 experiments. **(C)** Thrombi washed with PPACK as in B and subsequently perfused with PRP containing 50 µg/mL Alexa 488-labeled fibrinogen *(green)* for 15 min at 300 s^{-1}, after which the thrombi were incubated with anti-fibrin mAb 59D8. *Left panel*, a representative thrombus image shows accumulation of Alexa 488-fibrinogen (*green*); *middle panel*, the same area showing staining for fibrin (mAb 59D8) followed by incubation with Alexa 633-secondary antibody (*red)*; and *right panel*, merging of green and red labels to highlight co-localization of fibrinogen and fibrin. n=14 experiments. **(D)** Thrombi were treated with PPACK and then perfused with a solution of 2.7 mg/mL unlabeled fibrinogen containing 100 µM PPACK for 15 min. After washing, thrombi were stained with mAb FPA for intact fibrinogen *(green)*. The image shown is taken at 4 µm from the bottom of the chamber **(a).** Horizontal **(b)** and vertical **(c)** cross-sections of the thrombus were taken at the positions shown by white lines. **(d)** 3D reconstruction of the thrombus. n= 3 experiments.

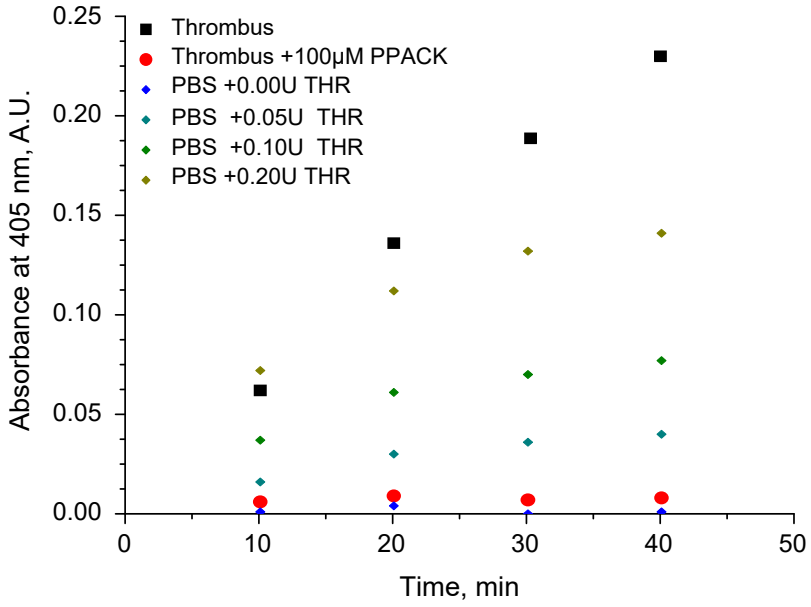


Figure 25. Thrombin activity associated with fibrin-rich thrombi. Thrombi generated at 3000 s^{-1} were washed with Tyrode buffer alone or supplemented with 100 μM PPACK at 300 s^{-1} for 15 min. The chamber was disassembled, washed with PBS, and the coverslip was placed in a 35 mm Petri dish containing 2 mL of 25 μM S-2238. The dishes were incubated at 37 °C and absorbance at 405 nm was determined at 10-30 min. Control samples were performed with 0.05 and 0.1 U/mL of thrombin. n=3 experiments.

Even though colocalization of Alexa 488-labeled fibrinogen with fibrin in the experiments described above (Fig. 24C) is an indicator of the newly deposited material, the Alexa 488 label on its own cannot discriminate between intact fibrinogen and fibrin since part of the labeled fibrinogen could be converted into fibrin. Therefore, we sought to verify that Alexa 488-associated protein was intact fibrinogen. The generated thrombi (Fig. 26A) were washed and then allowed to rest for an additional 15 min in the flow chamber to allow conversion of surface-associated fibrinogen into fibrin. Subsequently, Alexa 488-labeled fibrinogen (green) in Tyrode buffer + PPACK (100μM) was perfused over the thrombi, which were then stained for intact fibrinogen with mAb FPA (red). As shown in Fig. 26B, a significant amount of fibrinogen was adsorbed on the surface of thrombi and the majority of this protein was intact (Fig. 26C). The intact state of fibrinogen was further confirmed by the colocalization of the two labels (Fig. 26D). Inspection of their distribution also showed that there were small areas where only the green label (Alexa-488 labeled fibrinogen) was observed (Fig. 26D-E, boxed areas), indicating the presence of residual thrombin activity resulting in the conversion of Alexa 488-labeled fibrinogen into fibrin. The addition of other thrombin inhibitors including hirudin (2.5 μM) or argatroban (200 μM) during perfusion with Alexa 488-labeled fibrinogen produced similar results. Together, these data indicate that intact fibrinogen is deposited on the surface of preformed thrombi and its quantity depends on the presence of thrombin activity.

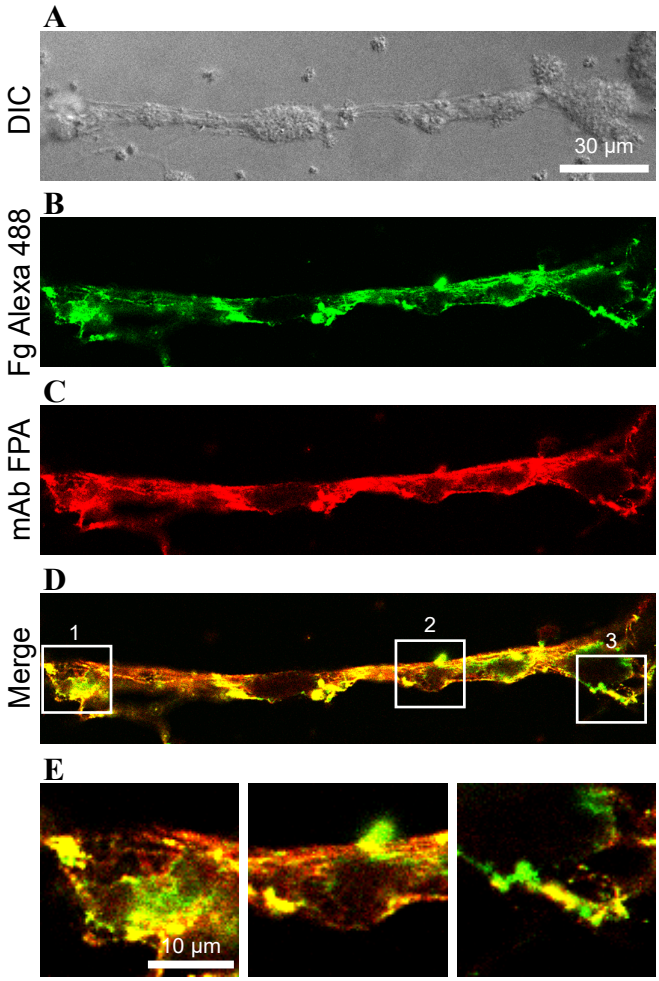


Figure 26. Detection of intact fibrinogen on the surface of fibrin-rich thrombi. Thrombi generated at shear rate of 3000 s^{-1} were allowed to rest in Tyrode buffer for an additional 15 min to convert surface-associated fibrinogen into fibrin. Subsequently, Alexa 488-labeled fibrinogen in Tyrode buffer containing 100 µM PPACK was perfused at 1500 s^{-1} for 15 min. **(A)** Representative DIC image of thrombi and connecting fibrin fibers. **(B)** Thrombi perfused with 20 µg/mL fibrinogen containing 1:100 Alexa 488-labeled fibrinogen *(green)*. **(C)** Thrombi were subsequently stained with mAb FPA *(red)*. **(D)** Fluorescent images of fibrinogen and fibrin merged to highlight colocalization of the two labels. **(E)** Enlarged images of the boxed areas shown in D. n=3 experiments

Platelet adhesion to the surface of thrombi displaying intact fibrinogen or fibrin. Having established conditions for the generation of stabilized thrombi covered with intact fibrinogen, we sought to verify that the fibrinogen coat protects thrombi from platelet adhesion. Three types of surfaces were prepared: "Fibrinogen", "Fibrin" and "Fibrin+PPACK" (described in Materials and methods). Subsequently, calcein-labeled platelets in Tyrode buffer were perfused over thrombi. Thrombi were then stained for fibrin (red) and fibrinogen (green) with the respective mAbs. As shown in Figure 27A (upper panel), when thrombin was inhibited by PPACK to halt the conversion of fibrinogen deposited on the surface of fibrin, no platelets were detected on the surface of thrombi. The presence of fibrinogen on the surface of fibrin was confirmed by staining of thrombi for fibrinogen and fibrin (Figs. 27A and 27D). In contrast, pronounced accumulation of platelets (blue) was seen on the surface of thrombi and connecting fibrin fibers when fibrin-bound fibrinogen was allowed to convert into fibrin (Fig. 27C, upper panel, and 27E). After blocking fibrin-bound thrombin with PPACK, a considerable quantity of platelets (~60%) adhered to fibrin, suggesting that although fibrin-bound thrombin enhanced adhesion perhaps by activating platelet receptors, nonstimulated platelets were still able to adhere (Fig. 27B). Platelet adhesion to both "Fibrin" surfaces was largely mediated by $\alpha_{IIb}\beta_3$ as anti-mAb 7E3 at 10 μg/ml inhibited adhesion by ~90%. Quantification of platelet adhesion showed that ~12- and 20-fold more platelets adhered to "Fibrin+PPACK" and "Fibrin" surfaces, respectively, than to the "Fibrinogen" surface (Fig. 27F). Since the presence of red blood cells (RBCs) may affect platelet adhesion, we perfused platelets over preformed thrombi in the presence of RBCs (25-50%). No increase in platelet adhesion to thrombi coated with fibrinogen was found.

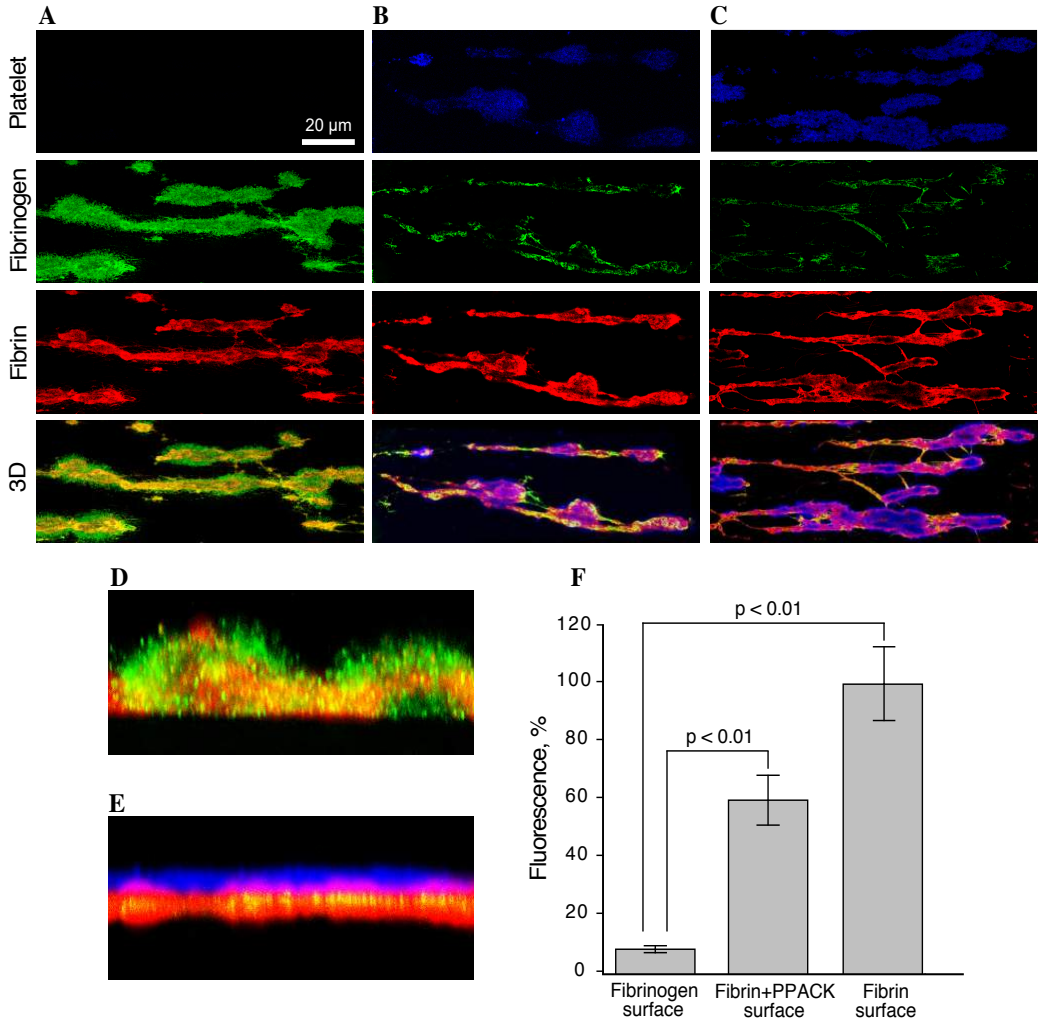


Figure 27. Deposition of platelets on the surface of thrombi expressing intact fibrinogen or fibrin. Thrombi generated at 3000 s^{-1} were incubated with PPACK (**A**), thrombin+PPACK (**B**) or thrombin only (**C**) to prepare the "Fibrinogen" (n=7 experiments), "Fibrin+PPACK" (n=5) or "Fibrin" (n=5) surfaces, respectively, as described in Materials and Methods. Calcein-labeled platelets *(blue)* in Tyrode buffer were perfused at 1500 s^{-1} for 15 min (A-C, *upper panels*). Thrombi incubated with mAb 59D8 for fibrin *(red)* or mAb FPA for fibrinogen *(green)*. The fourth row from the top in each column shows merging of fluorescent images for fibrin and fibrinogen in A *(orange)* and that for fibrin and platelets in B and C *(purple)*. **(D) and (E)** vertical cross-sections of representative thrombi generated under conditions shown in A and C, respectively. **(F)** Platelet adhesion was quantified using ImageJ software and expressed as a percentage of maximal fluorescence observed in C ("fibrin" surface). Fluorescence of platelets was measured from 254x254 μm random fields of 40 representative images. Fluorescence of fibrin in each field was determined and used to normalize the number of adherent platelets. Statistical analysis was performed using one-way ANOVA followed by Tukey's test to determine significance ($p < 0.01$).

Furthermore, pre-incubation of platelets with ADP (10 μM) to trigger activation before their perfusion did not induce their adhesion to the surface of fibrinogen-coated thrombi, although single platelets and platelet aggregates were observed adherent to the collagen substrate (Fig. 28). It should be noted that our system models a step of thrombogenesis at which fibrin-rich thrombi have been stabilized and the generation of platelet agonists has ceased. Therefore, ADP was used only as a means to convert $\alpha_{IIb}\beta_3$ into its active form. Together, these results indicate that the deposition of fibrinogen on the surface of fibrin associated with thrombi prevents adhesion of either resting or activated platelets.

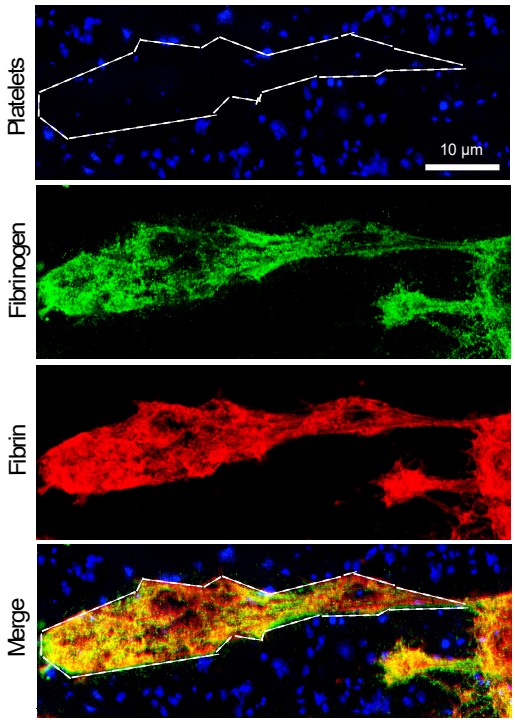


Figure 28. ADP-stimulated platelets on the surface of fibrin-rich thrombi expressing intact fibrinogen. Thrombi generated at a shear rate of 3000 s^{-1}, followed by 15 min perfusion of Tyrode buffer containing 100 μM PPACK at 1500 s^{-1}. After 15 min resting in the same perfusion buffer, a solution of calcein-labeled platelets (blue) supplemented with ADP (10 μM) was perfused at 1500 s^{-1} for 15 min. Thrombi were stained for fibrinogen (green) using mAb FPA and for fibrin (red) using mAb 59D8. The outline of the thrombus (white dashes) surrounded by adherent platelets is shown in the upper panel. n= 3 experiments.

DISCUSSION

In previous studies we identified a process in which fibrinogen adsorbed on various surfaces, including fibrin, dramatically reduces adhesion of platelets and leukocytes, and determined the molecular basis for this phenomenon (V. K. Lishko et al., 2007; I. S. Yermolenko et al., 2010; Jirouskova, Jaiswal, & Coller, 2007). However, these studies had been performed using pure fibrin clots formed under static conditions and with isolated blood cells. As for thrombi formed in flowing blood, they have a distinctive composition and architecture. Moreover, fibrin deposited around the platelet plug incorporates other blood proteins that may modify its fibrinogen binding capacity. Therefore, it was mandatory to show the presence of intact fibrinogen deposited on the surface and around fibrin-rich thrombi and to investigate its anti-adhesive properties. The major finding of this study is that thrombi generated from blood flowing over collagen-containing surfaces are coated with intact fibrinogen deposited on the boundary layer of thrombus-associated fibrin and on fibrin fibers around thrombi. This fibrinogen shell renders thrombi non-adhesive for platelets (schematically depicted in Fig. 29). These results provide direct experimental evidence for the presence of intact fibrinogen on the surface of *in vitro* thrombi and suggest that the fibrin cap, in addition to serving as a mechanical scaffold, functions as a platform for the self-assembly of the non-adhesive fibrinogen matrix.

Since fibrin is essential for stabilization of fibrin/platelet thrombi, numerous studies using *in vivo* models of thrombosis have examined its deposition (Jorgensen, Rowsell, Hovig, & Mustard, 1967; Hatton, Ross, Southward, DeReske, & Richardson, 1998; van Ryn et al., 2003; Groves et al., 1982; Kinlough-Rathbone et al., 1983; Hatton, Ross,

Southward, Timleck-DeReske, & Richardson, 2002; Steele et al., 1985). It was also shown that while platelet accumulation peaked within 30 min to one hour of arterial injury and then ceased(Groves, Kinlough-Rathbone, Richardson, Moore, & Mustard, 1979; Kelly et al., 1991; Barstad, Kierulf, & Sakariassen, 1996), fibrin formation continued for several hours (van Ryn et al., 2003). Remarkably, no further platelet accumulation onto the injury site has been observed when labeled platelets were injected several hours following injury (van Ryn et al., 2003). Likewise, *in vitro* models, in which platelet accumulation and fibrin formation were induced either on sections of an excised vessel wall or on substrates that mimic the sub-endothelial matrices, showed fibrin deposition that initially increased and then gradually stabilized (Weiss, Turitto, & Baumgartner, 1986; Gast, Tschopp, & Baumgartner, 1994; Mailhac et al., 1994; Berny et al., 2010; S. Falati et al., 2002). Two recent studies in living mice have shown that as the thrombus grows, its surface, consisting primarily of platelets, changes to that composed of fibrin (Kamocka et al., 2010; Cooley, 2011). However, fibrin overlaying platelet thrombi is not a universal finding among intravital studies in mice. The reason why some intravital investigations were able to detect a fibrin coat on the luminal thrombus surface (Kamocka et al., 2010; Cooley, 2011) whereas other studies detected fibrin only within and around thrombi (Hayashi et al., 2008; T. J. Stalker et al., 2013) and in the extravascular space underlying the thrombus (Hayashi et al., 2008; Kuijper, Gallardo Torres, Lammers, et al., 1997) is unclear. Differences in the methods used to induce thrombi, the times to monitor the course of thrombogenesis, the nature of vascular beds, and specificity of mAbs employed to detect fibrin may account for these discrepancies.

The *in vitro* model thrombi produced in our study show a strong resemblance, both in the overall morphology and the presence of a fibrin coat, to those produced *in vivo* (Kamocka et al., 2010; Cooley, 2011) and thus may represent a suitable model of fibrin-rich thrombi. The characteristic architecture of platelet-fibrin thrombi with a central platelet core and fibrin fibers aligned in the direction of flow (Gersh, Edmondson, & Weisel, 2010; Vandendries, Hamilton, Coughlin, Furie, & Furie, 2007) suggests that this structure may have specific functions. Consequently, only those *in vitro* thrombi that are generated from flowing blood and under conditions that allow for both the development of platelet thrombi and blood coagulation may be an appropriate model for studies of thrombus growth and dissolution. In contrast, a homogeneous structure of clots generated from platelet-rich plasma or pure fibrin under static conditions may be less informative. Nevertheless, while the *in vitro* perfusion chamber thrombosis model used in this as well as in many previous studies allows for the generation of thrombi with some morphological similarities to those formed *in vivo*, this reductive system clearly has many limitations. Thus, further studies of thrombi generated on excised vessel walls or in animal models of thrombosis may help to define the role of fibrin as a substrate for the deposition of the anti-adhesive fibrinogen layer.

Based on the fact that the fibrin cap is a feature of stabilized thrombi, it has been proposed that fibrin limits thrombus growth by physically separating pro-coagulant platelets from coagulation zymogens in blood (Kamocka et al., 2010). Although it is possible that fibrin may preclude the contact of circulating coagulation factors with the pro-coagulant platelet surface, it is also known to be a highly adhesive substrate for both resting and activated platelets mediating their attachment via integrin $\alpha_{IIb}\beta_3$ (Hantgan,

Hindriks, Taylor, Sixma, & de Groot, 1990; Jen & Lin, 1991; Hantgan et al., 1992; Endenburg, Hantgan, Sixma, de Groot, & Zwaginga, 1993). Therefore, if fibrin on the surface of stabilized thrombi was not shielded, it would remain thrombogenic, continually attracting platelets. Indeed, we show that when thrombi were manipulated to display fibrin (Figs. 27B, 27C, 27E and 27F) platelets readily accumulated on such surfaces. In contrast, no platelets were detected on the surface of thrombi that contained intact fibrinogen (Figs. 27A and 27D). Moreover, when platelets were tracked by time-lapsed video microscopy, they contacted the surface of thrombi only for a short period of time (Fig. 23), presumably because of the non-adhesive fibrinogen coat assembled on the surface of fibrin. This is in keeping with *in vivo* studies that did not detect platelets above the fibrin cap (Kamocka et al., 2010; Cooley, 2011) and in agreement with a finding that thrombi generated in *Par 4*$^{-/-}$ mice, which showed a robust fibrin accumulation, had no platelets adherent to the topmost surface of the fibrin clot (Sakariassen et al., 1990). It is also consistent with previous reports showing that no platelets have been observed on the surface of fibrin produced in *ex vivo* models of thrombosis (Weiss, Turitto, & Baumgartner, 1986; Kirchhofer, Tschopp, Steiner, & Baumgartner, 1995; Sakariassen et al., 1990).

Discussion of the mechanisms that are responsible for the cessation of thrombus growth has traditionally focused on those that halt the accrual of platelets to the platelet plug (D. A. Meh, Siebenlist, & Mosesson, 1996). However, it is the deposition of fibrin around the initial platelet plug and in its vicinity, as documented in numerous studies, that appears to be the end stage of thrombus formation. Therefore, the anti-adhesive mechanisms that prevent the accumulation of blood cells on this highly adhesive and,

thus, dangerous substrate should also play an important role in the control of thrombus propagation. The long-held view that fibrin serves as a mechanical scaffold stabilizing the thrombus structure is supported by numerous *in vitro* and *in vivo* studies. Our results indicate that fibrin may perform yet another important function by serving as a platform for the assembly of the non-adhesive fibrinogen layer. It is also possible that the fibrinogen matrix deposited atop of the fibrin cap may separate fibrin-bound thrombin, which remains enzymatically active (Berny et al., 2010; D. A. Meh, Siebenlist, & Mosesson, 1996; Fredenburgh, Stafford, Leslie, & Weitz, 2008) from the flowing blood. Although thrombin would obviously convert first arriving fibrinogen molecules into fibrin, this newly formed fibrin will not contain thrombin. Therefore, subsequent fibrinogen molecules that will interact with this thrombin-free fibrin will remain intact and initiate the formation of the fibrinogen multilayer through the interaction between the αC domains of the neighboring molecules (I. S. Yermolenko et al., 2010).

In conclusion, our in *vitro* model of fibrin-rich thrombi, formed under shear and flow conditions, were coated with intact fibrinogen that strongly reduced platelet adhesion. While there is little doubt that fibrinogen, upon its contact with fibrin, undergoes self-assembly, the exact structure of the fibrinogen matrix remains to be determined. The nanoscale nature of the fibrinogen multilayer presents a formidable challenge for studies of this structure deposited on the surface of fibrin. At present, even state-of-the art microscopy methods such as high-resolution AFM-based imaging are not able to reveal the molecular structure of the fibrinogen matrix deposited on the soft fibrin polymers. Therefore, the conclusions and models generated in our previous studies of fibrinogen

deposition on hard surfaces such as mica or glass (I. S. Yermolenko et al., 2010) should provide guidance for future analyses. Likewise, our present studies and previous low resolution demonstration that fibrinogen accumulates in a thin superficial layer of fibrin clots may guide future analyses of the spatial distribution of fibrinogen and other proteins on the flow surface of thrombi *in vivo*.

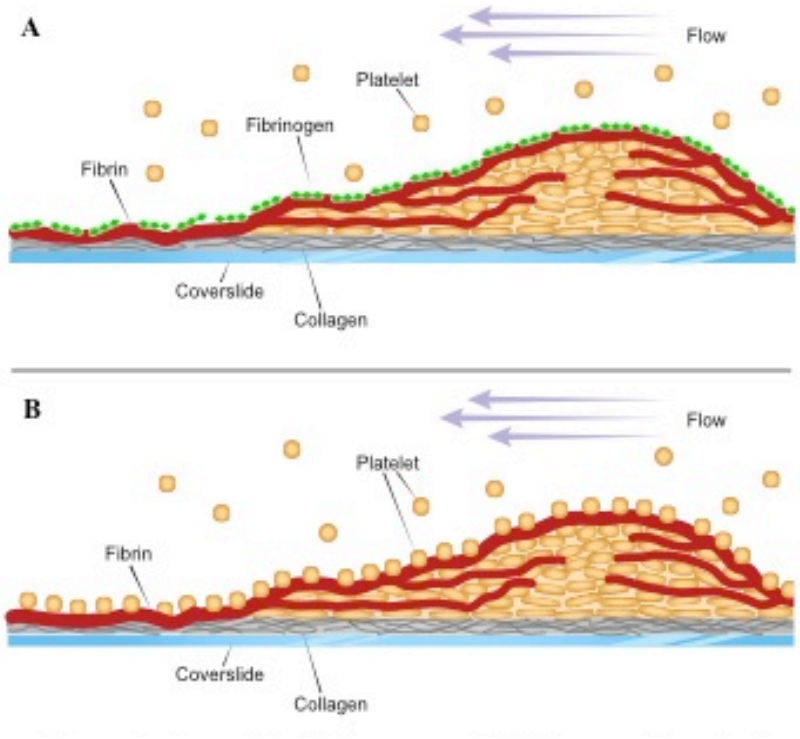


Figure 29. A model of the proposed fibrinogen-dependent anti-adhesive mechanism operating on the surface of stabilized fibrin-rich thrombi. **(A)** When fibrin on the surface of a thrombus is covered with the fibrinogen matrix (green diamonds), platelets do not adhere. **(B)** In contrast, platelets accumulate on the surface of a thrombus covered with fibrin (red lines). Blood flow is shown from right to left (purple arrows).

CHAPTER 5

FIBRINOGEN MATRIX DEPOSITED ON THE SURFACE OF BIOMATERIALS ACTS AS A NATURAL ANTI-ADHESIVE COATING

ABSTRACT

Adsorption of fibrinogen on the luminal surface of biomaterials is a critical early event during the interaction of blood with implanted vascular graft prostheses and determines their thrombogenicity. We have recently identified a nanoscale process by which fibrinogen modifies the adhesive properties of various surfaces for platelets and leukocytes. In particular, adsorption of fibrinogen at low density promotes cell adhesion while its adsorption at high-density results in the formation of an extensible multilayer matrix, which dramatically reduces cell adhesion. It remains unknown whether deposition of fibrinogen on the surface of vascular graft materials produces this anti-adhesive effect. Using atomic force spectroscopy, single cell force spectroscopy, and standard adhesion assays with platelets and leukocytes, we have characterized the adhesive and physical properties of the contemporary biomaterials, before and after coating with fibrinogen. We found that uncoated PET, PTFE and ePTFE exhibited high adhesion forces developed between the AFM tip or cells and the surfaces. Adsorption of fibrinogen at increasing concentrations progressively reduced adhesion forces, and at ≥ 2 μg/mL all surfaces were virtually non-adhesive. Standard adhesion assays performed with platelets and leukocytes confirmed this dependence. These results provide a better understanding of the molecular events underlying thrombogenicity of vascular grafts.

INTRODUCTION

It is widely recognized that after implantation, prosthetic vascular grafts spontaneously acquire a layer of adsorbed plasma proteins. Among them, fibrinogen has received the most attention because of its high concentration in the circulation (2-3 mg/mL), quick adsorption on various surfaces, and ability to support integrin-mediated adhesion of platelets and leukocytes. Since one of the primary graft failures is adhesion-dependent platelet activation and thrombus formation, fibrinogen is generally viewed as a culprit. Consequently, considerable research effort has been made to create nonfouling materials that resist protein adsorption and platelet adhesion (reviewed in (Jordan & Chaikof, 2007; Yang, Xue, Li, Zhang, & Jiang, 2009; Pillai et al., 2009). In addition, innumerable *in vitro* studies have focused on the mechanisms of fibrinogen adsorption in an attempt to understand its thrombogenic potential (Lindon, McManama, Kushner, Merrill, & Salzman, 1986; W. B. Tsai, Grunkemeier, & Horbett, 1999; W. B. Tsai, Grunkemeier, & Horbett, 2003; Wu, Simonovsky, Ratner, & Horbett, 2005; Xu & Siedlecki, 2009; Sivaraman & Latour, 2010; Szott & Horbett, 2011) and references therein]. Paradoxically, other studies have focused on the design of anti-thrombogenic vascular grafts utilizing fibrin(ogen)-coated surfaces with apparently beneficial results in animal studies and in humans (Patel et al., 1992; Gosselin, Ren, Ellinger, & Greisler, 1995; Swartz, Russell, & Andreadis, 2005; Hasegawa, Okada, Takano, Hiraishi, & Okita, 2007).

Intact fibrinogen deposited on various surfaces is indeed adhesive for platelets and leukocytes, but only when adsorbed at certain concentrations and, especially, when cell adhesion is tested in buffers without fibrinogen (Coller, 1980; B. Savage & Ruggeri,

1991; Endenburg et al., 1993; Endenburg et al., 1996; V. K. Lishko et al., 2007). Furthermore, fibrin, a clotting product of fibrinogen, mediates strong adhesion of blood cells suspended in protein-free buffers (Endenburg et al., 1993) (V. K. Lishko et al., 2007; Jen & Lin, 1991; van Ryn et al., 2003). From these *in vitro* observations, it is expected that fibrinogen, which is deposited early after vascular graft implantation, should support rapid accumulation of platelets. Likewise, fibrin, which is eventually formed and remains on the surface of grafts implanted in humans over the years, should promote platelet deposition and activation. This, in turn, would be expected to result in continuous thrombus propagation and vascular occlusion. However, the flow surface of vascular grafts is highly thrombogenic only during the early postoperative period (Hamlin, Rajah, Crow, & Kester, 1978; Goldman, Norcott, Hawker, Hail, et al., 1982; Goldman, Norcott, Hawker, Drolc, & McCollum, 1982). Subsequently, graft maturation results in a non-thrombogenic surface (Goldman, Norcott, Hawker, Drolc, & McCollum, 1982; Harker, Slichter, & Sauvage, 1977; McCollum, Kester, Rajah, Learoyd, & Pepper, 1981), which sustains its characteristic acellular appearance over the decades in spite of the presence of the stable fibrin lining (De Bakey, Jordan, Abbott, Halpert, & O'Neal, 1964; Szilagyi, Smith, Elliott, & Allen, 1965; Berger, Sauvage, Rao, & Wood, 1972; Shi et al., 1997). These observations suggest the existence of natural anti-adhesive mechanisms operating on the flow surface of mature vascular graft prostheses that prevent the accumulation of blood cells. The crucial question as to why platelets adhere to the fibrinogen layer deposited on the surface of vascular grafts early after implantation but do not attach to the highly adhesive fibrin formed on mature grafts remains unaddressed.

In recent reports we described a new nanoscale phenomenon whereby adsorption of fibrinogen on various surfaces, including fibrin clots, dramatically reduces cell adhesion. In particular, we showed that the ability of fibrinogen to support cell adhesion strikingly depends on its coating concentration. Counter-intuitively, while low-density fibrinogen is highly adhesive for platelets and leukocytes, its adsorption at the increasing concentrations reduces cell adhesion under both static and flow conditions (V. K. Lishko et al., 2007; Jen & Lin, 1991; van Ryn et al., 2003). This effect is specific for fibrinogen since no other plasma proteins exhibit this unusual behavior (V. K. Lishko et al., 2007). The underlying mechanism by which fibrinogen renders surfaces non-adhesive is its surface-induced unfolding and self-assembly leading to the formation of a nanoscale (~10 nm thick) multilayer matrix. The fibrinogen matrix is extensible and characterized by weak adhesion forces, which makes it incapable of transducing strong mechanical forces via cellular integrins, resulting in weak intracellular signaling and infirm cell adhesion (I. S. Yermolenko et al., 2010; I. S. Yermolenko et al., 2012). In contrast, fibrinogen deposited at low concentrations produces a single molecular layer in which molecules are directly attached to the surface. The monolayer is characterized by high adhesion forces that transduce strong outside-in signaling in platelets leading to firm adhesion. It remains unknown whether this behavior is observed when fibrinogen is adsorbed on the surface of vascular graft materials.

In this study, employing atomic force microscopy (AFM)-based force spectroscopy, single cell force spectroscopy (SCFS) and standard adhesion assays with platelets and leukocytes, we examined the adhesive and physical properties of two materials, PTFE (polytetrafluoroethylene) and PET (polyethylene terephthalate) used for

the production of contemporary vascular grafts, before and after adsorption of fibrinogen. We show that in spite of the high adhesion forces exhibited by uncoated biomaterials and when coated with low fibrinogen concentrations, adsorption of fibrinogen at higher concentrations renders the surfaces virtually non-adhesive for platelets and leukocytes.

MATERIALS AND METHODS

Materials. Human fibrinogen depleted of fibronectin and plasminogen was obtained from Enzyme Research Laboratories (South Bend, IN). Fibrinogen was treated with iodoacetamide to inactivate the residual Factor XIII and then dialyzed against phosphate buffered saline (PBS). Fibrinogen was labeled with 125-Iodine using IODO-GEN (Thermo Scientific Pierce Protein Research Products, Rockford, IL), dialyzed against PBS and stored at -20 °C. The concentration of fibrinogen was determined by spectrophotometry at 280 nm using the extinction coefficient 1.51 at 1 mg/mL. Polyclonal antibodies against human fibrinopepetide A was obtained from Assypro (St. Charles, MO). Calcein AM was purchased from Molecular Probes (Life Technologies, Grand Island, NY). The following biomaterials were used in this study: (1) Mylar™, a non-crystalline form of polyethylene terephthalate (PET) was purchased as a film (type A, 0.127-mm thick) from Cadillac Plastic and Chemical (Birmingham, MI); (2) polytetrafluoroethylene (PTFE) was obtained as a 0.0508-mm sheet from Professional Plastics (Fullerton, CA); and (3) Impra® ePTFE (expanded PTFE; intermodal distance 30 μm) vascular graft was obtained from Bard Peripheral Vascular Inc. (Tempe, AZ) in the form of vascular graft tubing with a diameter of 7 mm.

Cell culture. Human embryonic kidney 293 cells stably expressing leukocyte integrin Mac-1 (HEK-Mac-1) have been described previously (Yakubenko, Lishko, Lam, & Ugarova, 2002; Valeryi K Lishko, Yakubenko, & Ugarova, 2003). The cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin. Before each experiment, cells were detached from the flasks using cell dissociation buffer (Cellgro, Mediatech Inc., Manassas, Virginia), washed twice in Hank's Balanced Salt solution (HBSS) containing Ca^{2+} and Mg^{2+} and resuspended in HBSS + 0.1% BSA. U937 monocytic cells were obtained from the American Tissue Culture Collection and cultured in RPMI 1640 supplemented with 10% fetal bovine serum.

Platelets isolation. Human platelets were isolated as described previously (Ugarova et al., 1993). Blood was obtained via venipuncture from healthy volunteers who had not taken any aspirin in the previous two weeks. Briefly, the collected blood was anti-coagulated with acid citrate dextrose (ACD) in the presence of 2.8 μM prostaglandin E1 (PGE1). Platelets were obtained by differential centrifugation, followed by gel-filtration on Sepharose 2B-CL in calcium-free Tyrodes buffer containing 0.1% bovine serum albumin (BSA) and 0.1% glucose, pH 7.2.

Surface preparation for AFM and SCFS. The PET and PTFE sheets were cut into small squares and rinsed with methanol, ethanol, and deionized water. The ePTFE samples were prepared from the vascular graft tubing by cutting squares of the same size. The samples for AFM and SCFS were cut into 6x6 mm squares and prepared by

adsorption of different concentrations of fibrinogen (0.05-20 μg/mL) in PBS onto the biomaterial surfaces for 3 h at 37 °C.

Atomic force microscopy (AFM)-based force spectroscopy. Force-distance measurements on fibrinogen coated substrates of biomaterials were performed in PBS at 37 °C using an MFP-3D atomic force microscope (Asylum Research, Santa Barbara, CA) as described (I. S. Yermolenko et al., 2010; I. S. Yermolenko et al., 2012). Silicon nitride probes (MLCT, Veeco Probes, Camarillo, CA) with nominal spring constants in the range of 10-15 pN/nm were used. Cantilevers were plasma-cleaned in an oxygen plasma for 40 sec before use. Surfaces coated with different concentrations of fibrinogen were probed using a force trigger of 600 pN and an approach and retract speed of 2 μm/s. Force-distance curves were acquired in 64x64 arrays of 5x5 μm^2 areas. For each concentration of fibrinogen three arrays at different areas on the surface were probed (12288 force-distances per coating concentration). Spring constants for each cantilever were determined using the built-in Thermal method. Each curve was analyzed by a custom program written in IGOR Pro 6 (Wavemetrics, Lake Oswego, OR), which calculates adhesion forces. To obtain mean values of these parameters, the data were fitted using log-normal distributions.

Single cell force spectroscopy (SCFS). Cantilevers for SCFS measurements were prepared as previously described (I. S. Yermolenko et al., 2010). Briefly, tipless cantilevers with nominal spring constants of 0.035 N/m (HYDRA6R-200NG-TL, AppNano, Santa Clara, CA) were functionalized with (3-aminopropyl) triethoxysilane

(APTES). After washing, the cantilevers were incubated in 1.25 mM Bis (ssulfosucccinimidil) suberate (BS^3; Sigma) solution for 30 min at 22 °C and then placed into 0.5 mg/mL concanavalin A (Sigma) solution for 30 min at 22 °C. Cantilevers were then rinsed with PBS and stored in 1 M NaCl at 4 °C. SCFS measurements were performed in HBSS with 0.1% BSA at 37 ^{o}C. Integrin Mac-1-expressing HEK293 cells were pipetted into the custom-made AFM chamber and a single cell was instantly picked up from the glass surface by pressing on a cell with a cantilever using a contact force of 500-2000 pN for 5-30 sec. After that, the cell was lifted from the surface, allowed to attach firmly to the cantilever for 5-10 min and then lowered onto the fibrinogen coated biomaterial. The biomaterials coated with different concentrations of fibrinogen were probed using a 1 nN force trigger, an approach and retract speed of 2 μm/s, and a 120 s dwell time on the surface. After the trigger force was reached, the z position was kept constant. After each force measurement, a cell was allowed to recover for at least 5 min before the next cycle. As monitored optically, the cells always detached from the fibrinogen substrate but not from the cantilever during pulling. Cell detachment was indicated by the superposition of trace and retrace baselines over the pulling range (25-35 μm).

Fluorescence confocal microscopy. ePTFE was coated with fibrinogen (20 μg/mL) and incubated with calcein-labeled platelets for 30 min at 37 °C. The samples were stained with anti-fibrinogen mAb FPA followed by Alexa 647-conjugated goat anti-mouse secondary antibody. Confocal images were taken using a confocal microscope

(Leica TSC SP5, Buffalo Grove, IL) at excitation wavelengths of 488 (green: platelets) and 647 (red: fibrinogen).

Scanning electron microscopy (SEM). PET, PTFE, and ePTFE samples were precoated by a standard sputtering technique with Au-Pd alloy. Samples were observed using a JEOL JSM6300 (Tokyo, Japan) scanning electron microscope. In selected experiments, PET was coated with different concentrations of fibrinogen on which platelets ($1x10^7$/mL) were allowed to adhere. After 30 min incubation at 37°C, samples were washed, fixed with 2% gluteraldehyde, and post-fixed with 1% osmium tetroxide (OsO4) in 1x PBS (pH 7.4) for 1 h each at 22 °C. Samples were dehydrated and then dried through the critical point drying of CO_2, mounted onto aluminum stubs, and sputter coated with palladium-gold (approximately 5 nm) in a sealed vacuum. The samples were observed using a JEOL JSM6300 microscope.

Standard cell adhesion assays. The biomaterial surfaces were freshly cut into squares (9x9 mm), cleaned with methanol, ethanol and deionized water, and placed into 24-well plates. The biomaterials were incubated with different concentrations of fibrinogen for 3 h at 37 °C. 3 mL of calcein-labeled U937 cells (1×10^6/mL) or calcein-labeled platelets (1×10^7/mL) in Hanks' balanced salt solution (HBSS) or Tyrode buffer, respectively, containing 0.1% BSA were added to the surfaces. After a 30 min incubation at 37 °C, the non-adherent cells were removed by dipping the surfaces into PBS. Photographs of five random fields for each surface were taken with the 20x and 40x objectives of a Leica DM4000B microscope (Leica Microsystems, Buffalo Grove, IL),

and the number of adherent cells was counted. In selected experiments, adherent U937 cells were fixed with 4% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 5 min, then washed with PBS. Finally, cells were incubated with Alexa Fluor 546 phalloidin (5 μL of methanolic stock solution (~6.6 μM) diluted into 200 μL PBS) for 30 min at 22 °C to stain for the actin cytoskeleton.

Statistical analyses. Statistical significance of differences between mean values for various conditions has been assessed using a Student's *t*-test, with $p \leq 0.05$ considered as statistically significant. For SCFS, statistical significance between median values for different samples was evaluated using a Mann-Whitney U test, with $p < 0.05$ considered as statistically significant.

RESULTS

Characterization of selected biomaterials by SEM. To assess how fibrinogen might modify the adhesive properties of biomaterials, we have selected two commonly used materials: polyethylene terephthalate (PET or Dacron) and polytetrafluoroethylene (PTFE). Since an uneven surface of woven or knitted Dacron used in commercial grafts may complicate the AFM-based force spectroscopy analyses, we have used smooth flat sheets made of solid PET, which mimics the chemical and mechanical properties of the vascular graft material (Anamelechi, Truskey, & Reichert, 2005). Likewise, the flat sheets made of PTFE were shown to imitate the chemical properties of ePTFE used for the production of vascular tubes having a complex three-dimensional structure (Anamelechi, Truskey, & Reichert, 2005). Scanning electron microscopy of the test

materials showed the differences in the surface architecture of PET and PTFE (Fig. 30). The figure also shows the typical structure of ePTFE with irregular-shaped flat islands (known as "nodes") and a dense meshwork of fine fibers stretching between the nodes.

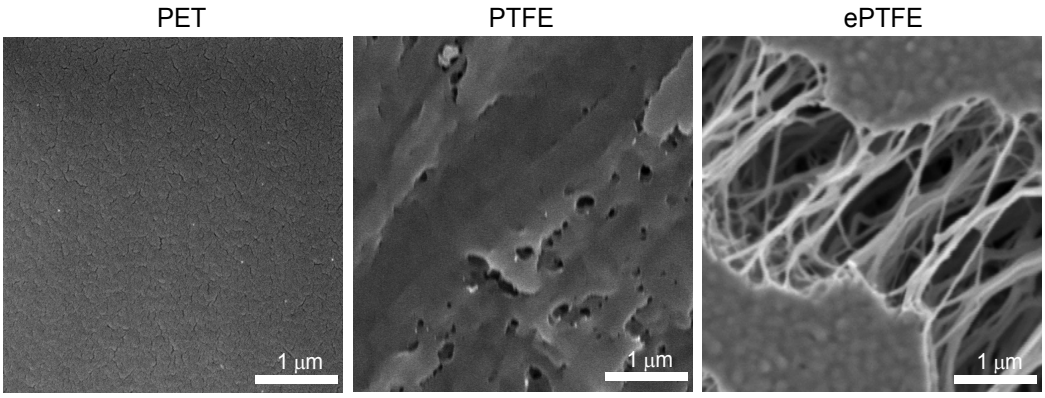


Figure 30. Scanning electron microscopy images of biomaterial surfaces. The PET, PTFE and ePTFE surfaces were prepared for SEM as described in Materials and Methods.

Adhesion Forces Developed by Uncoated and Fibrinogen-Coated Biomaterials Determined by Force Spectroscopy. Adhesion forces of the surfaces before and after adsorption of different concentrations of fibrinogen were probed using an unmodified AFM tip. The strongest adhesion forces were observed between the AFM tip and uncoated materials (Fig. 31A-C). Among the materials, both PET and PTFE displayed higher adhesion forces (~1100 pN) compared to ePTFE (~600 pN). Because of the complex architecture, the adhesion forces on ePTFE were measured on the surface of nodes and found to be 596±191 pN. The basis for the ~1.7-fold difference in adhesion forces between naked PTFE and ePTFE is not clear since both are made of chemically identical material, but potentially may arise from the ePTFE's modification during manufacturing.

As expected, the increase in the fibrinogen coating concentrations resulted in the decrease of adhesion forces for all three materials. In agreement with previous data obtained on mica and glass (I. S. Yermolenko et al., 2010), the steep decrease of adhesion forces occurred in a very narrow range of fibrinogen coating concentrations (0-1 mg/mL for PTFE and ePTFE) and 0-2 mg/mL for PET. At the concentrations ≥ 1-2 mg/mL, the adhesion forces reduced by ~6-, 40- and 3-fold for PET, PTFE, and ePTFE, respectively, compared to uncoated surfaces. Additional increase in the concentration of fibrinogen up to 50-100 mg/mL did not produce an additional decrease in the adhesion forces for PTFE, although it further decreased adhesion forces for PET and ePTFE. These results indicate that regardless of the initial adhesiveness of uncoated surfaces, the deposition of fibrinogen results in a dramatic drop of adhesion force making the surface of biomaterials virtually non-adhesive.

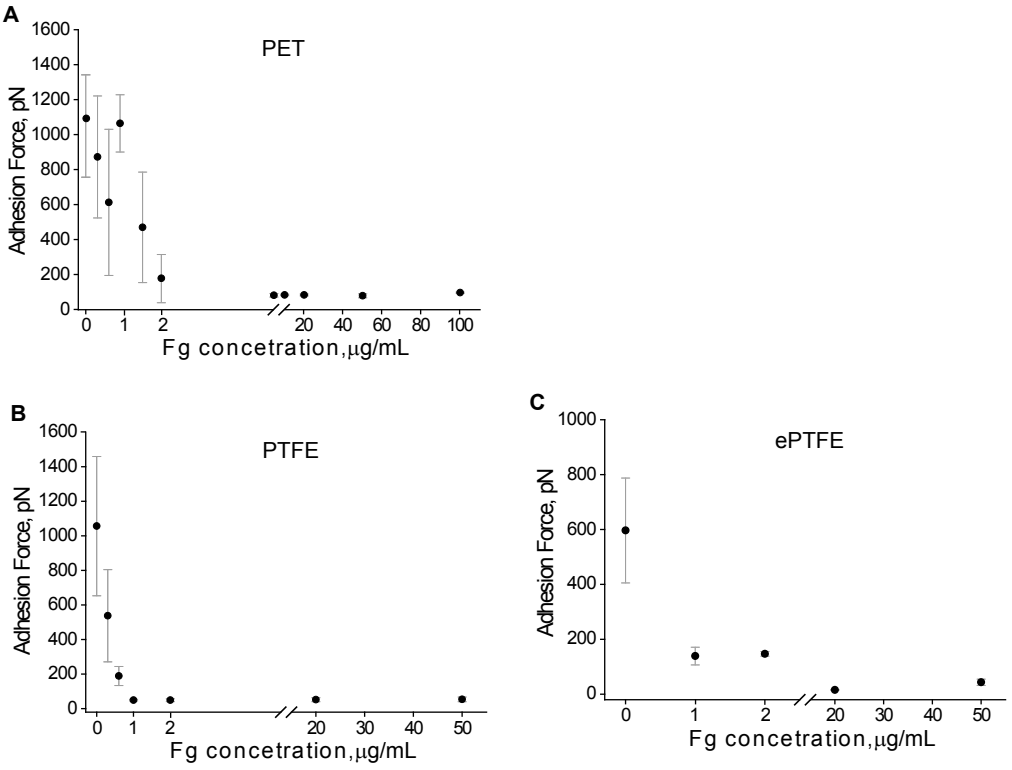


Figure 31. Force spectroscopy analyses of the surfaces prepared by adsorption of fibrinogen on biomaterials. Most probable adhesion forces between an unmodified AFM tip and fibrinogen coated biomaterials plotted as a function of the fibrinogen coating concentration adsorbed on PET **(A)**, PTFE **(B)** and ePTFE **(C)**. The data shown are the means ± SD from three to four experiments with 12288-16384 force-distance curves collected from the force maps for each concentration of fibrinogen. Fg: fibrinogen.

Adhesion of U937 monocytic cells and platelets to biomaterials examined by standard adhesion assays. We previously showed a parallelism between the adhesion forces probed by AFM-based force spectroscopy and cell adhesion tested in standard adhesion assays I. S. Yermolenko et al., 2010; I. S. Yermolenko et al., 2012). To examine whether adsorbed fibrinogen reduces integrin-mediated cell adhesion on biomaterials, we performed adhesion assays with platelets isolated from blood and U937 monocytic cells, a model cell line commonly used in lieu of peripheral blood monocytes. The surfaces were coated with increasing concentrations of fibrinogen and calcein-labeled platelets or U937 cells were allowed to adhere for 30 min at 37 °C. As shown in Figs. 32A and 32B for U937 cells and in 32C and 32D for platelets, cell adhesion progressively declined as the coating concentration of fibrinogen adsorbed on the surface of PET increased. Furthermore, while U937 cells and platelets were spread on low-density fibrinogen (1.0 mg/mL), they remained round on surfaces coated with 20 mg/mL fibrinogen, underscoring a direct relationship between cell spreading and firm adhesion (Fig. 32B and 32D). Similar results were obtained using flat PTFE sheets (Fig. 33).

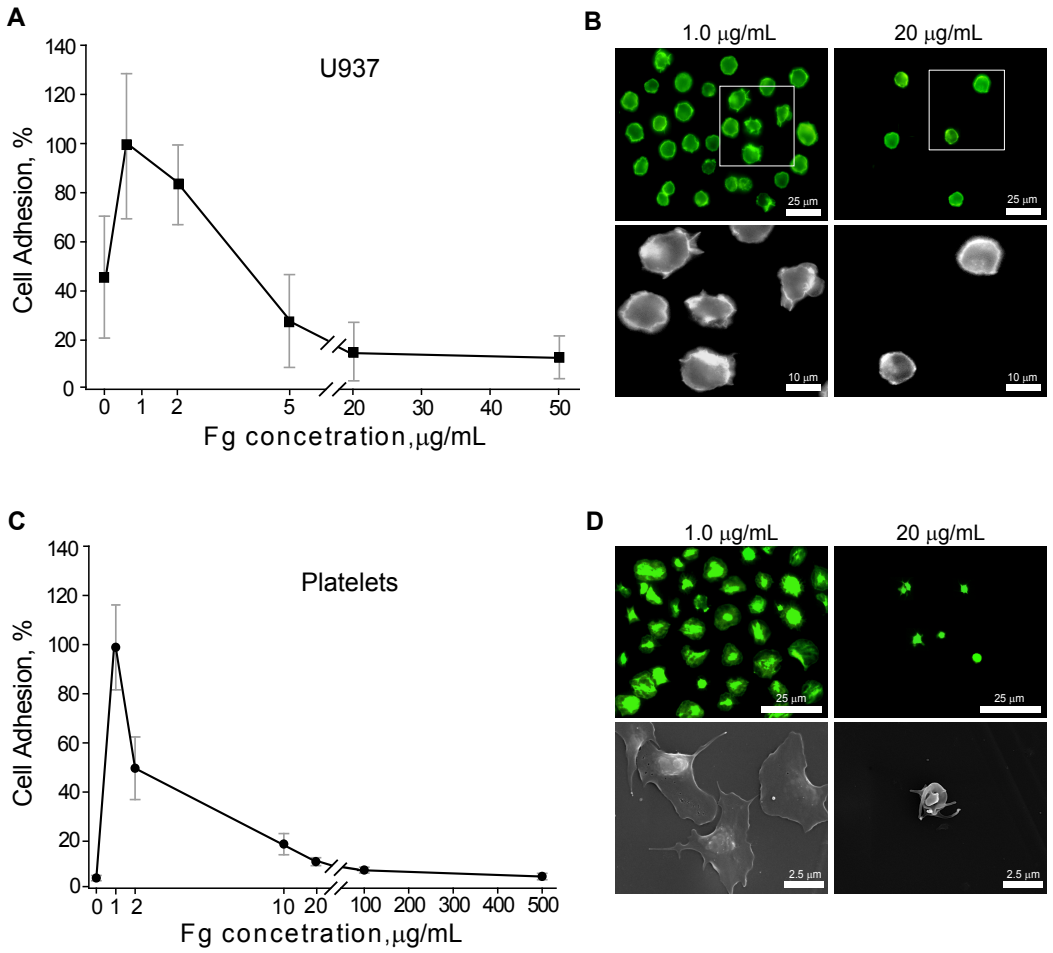


Figure 32. Adhesion of U937 monocytic cells and platelets to fibrinogen-coated PET. Different concentrations of fibrinogen were adsorbed on the surface of PET squares (9x9 mm) and calcein-labeled U937 cells **(A)** or platelets **(C)** were allowed to adhere for 30 min at 37 °C. Non-adherent cells were removed by washing, and adherent cells were quantified by taking pictures of five random fields with 20x and 40x objectives. Three PET surfaces were prepared for each fibrinogen concentration. Adhesion is expressed as a percentage of maximal adhesion observed at 1 μg/mL. Data shown are means ± SD of three independent experiments with each cell type. **(B)** Representative fluorescent images of U937 cells adherent to PET coated with 1 μg/mL (*left panels*) and 20 μg/mL (*right panels*) fibrinogen. Adherent U937 cells were fixed in 4% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 5 min, and then washed and incubated with Alexa Fluor 546 phalloidin to stain for the actin cytoskeleton**. (D)** Representative fluorescent (*upper panels*) or scanning electron microscopy (*lower panels*) images of platelets adherent to PET coated with 1 μg/mL (*left panels*) and 20 μg/mL (*right panels*) fibrinogen.

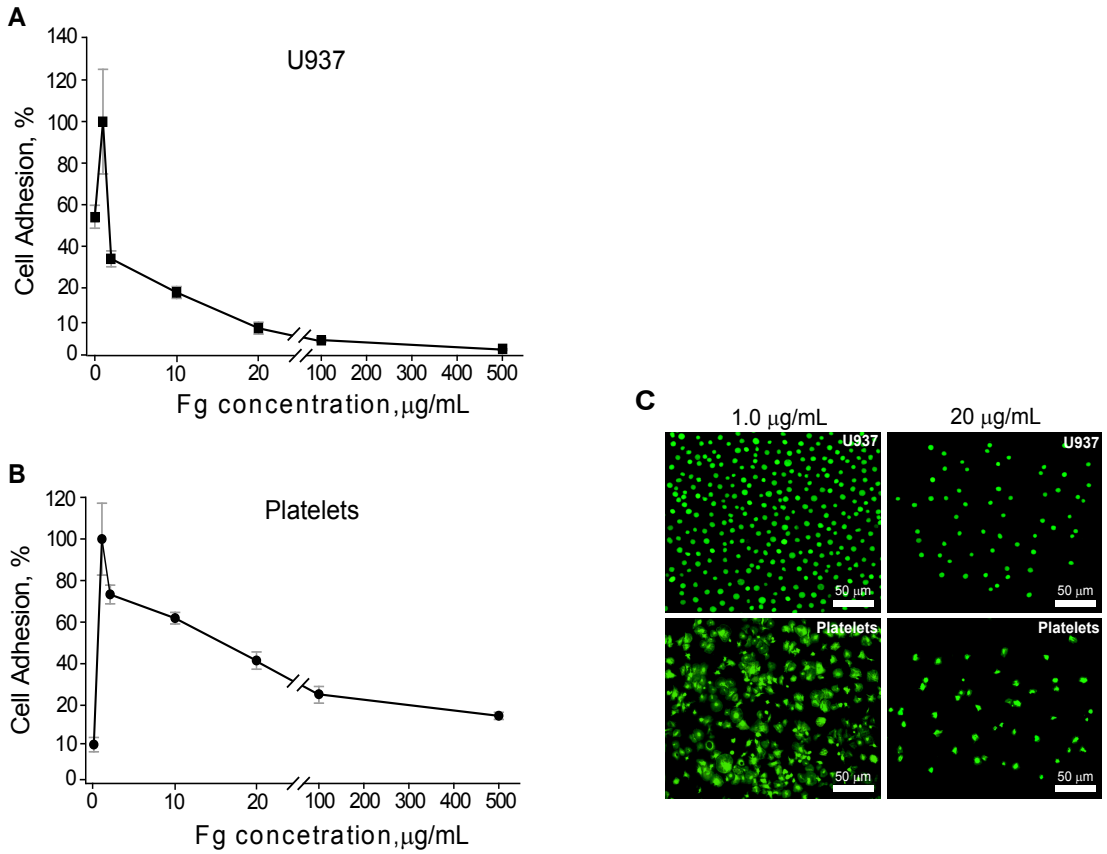


Figure 33. Cell adhesion to fibrinogen-coated PTFE. Calcein-labeled U937 cells **(A)** or platelets **(B)** were allowed to adhere for 30 min at 37 °C to PTFE squares (9x9 mm) coated with different concentrations of fibrinogen. Non-adherent cells were removed by washing and the number of adherent cells was quantified by taking pictures of five random fields, per each concentration of fibrinogen, with 20x and 40x objectives. Cell adhesion is expressed as a percentage of maximal adhesion observed at 1 μg/mL. Data shown are means ± SD of three independent experiments with each cell type. **(C)** Representative fluorescent images of U937 cells (*upper panel*) or platelets (*lower panel*) adherent to PTFE coated with 1 μg/mL (*left panel*) or 20 μg/mL (*right panel*) fibrinogen.

The lack of correlation between the coating concentration of fibrinogen and cell adhesion was not due to the abnormal adsorptive capacity of PET and PTFE. Using ^{125}I-labeled fibrinogen, we verified that deposition of protein on PET and PTFE followed a dose-dependent pattern (Fig. 34). Notable, however, was the clear lack of saturation of fibrinogen adsorption, which was observed on other surfaces (V. K. Lishko et al., 2007) and may be attributed to a continuing assembly of the multilayer. Adhesion assays with fibrinogen-coated ePTFE were hampered by the non-uniform nature of the surface. It was found that many platelets and U937 cells were mechanically trapped within the meshwork of fibers connecting the nodes. Nevertheless, the results revealed that only a few platelets adhered to the flat nodes (Fig. 35; shown for platelets). Thus, consistent with previous findings, fibrinogen adsorbed on the surface of biomaterials at low concentrations supports cell adhesion while high concentrations render the surfaces non-adhesive.

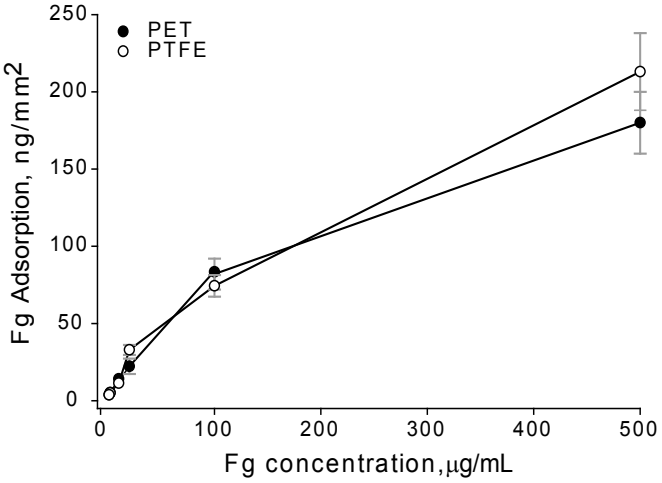


Figure 34. Adsorption of fibrinogen on biomaterial surfaces. The surfaces of PET and PTFE squares were incubated with different concentrations of ^{125}I-labeled fibrinogen (1-500 μg/mL) for 3 h at 37 °C. Excess fibrinogen was removed by washing the PET and PTFE squares with PBS. Bound fibrinogen was determined by measuring radioactivity using a gamma counter.

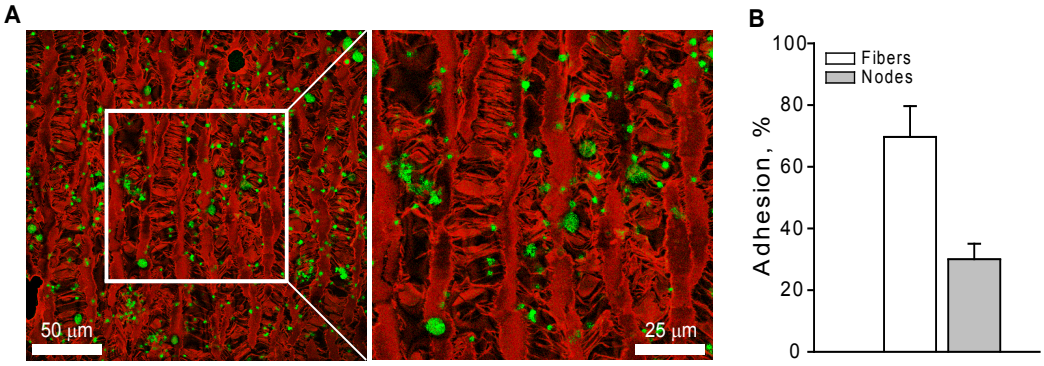


Figure 35. Platelet adhesion to fibrinogen-coated ePTFE material. ePTFE was coated with 20 μg/mL fibrinogen and incubated with calcein-labeled platelets. Non-adherent platelets were removed by washing with PBS. The samples were stained with an anti-fibrinogen polyclonal antibody that recognizes intact fibrinogen followed by an Alexa 647-conjugated goat anti-rabbit secondary antibody. **(A)** Platelets (green) are seen mainly enmeshed into fibers and not on the surface of nodes. **(B)** The number of platelets on the surface of nodes and trapped inside the fibers was quantified. Adhesion was expressed as a percentage of total platelet number in the sample.

Single-cell Force Spectroscopy (SCFS). Although standard adhesion assays provide useful insights into the capacity of various substrates to support cell attachment, these analyses are largely qualitative. Therefore, we obtained quantitative data by measuring the maximal adhesion forces developed between cells and coated biomaterials using SCFS. In these analyses we used HEK293 cells stably expressing leukocyte integrin Mac-1 (a_Mb_2). This cell line was previously employed to characterize integrin Mac-1-dependent adhesion and to measure adhesion forces between cells and fibrinogen-coated mica (I. S. Yermolenko et al., 2010). Single Mac-1-HEK cells were attached to a Con A-functionalized cantilever, and then each cell was brought into contact with uncoated or fibrinogen-coated PET and PTFE surfaces (Fig. 36A). Cells were pressed onto each surface with a force of 1 nN for 120 sec and then withdrawn with a constant

speed of 2 mm/s. The maximal adhesion forces were determined from force-distance retraction curves (Fig. 36B). Fig. 7C and 7D show box plots for the maximal forces required for cell detachment from uncoated PET and PTFE, respectively, and from the corresponding surfaces coated with low and high concentrations of fibrinogen. For uncoated PET, the median force for detachment was 773 pN. The 25^{th} percentile was 487 pN, and the 75^{th} percentile was 1001 pN (Fig. 36C). Adsorption of low concentrations of fibrinogen (0.3-0.6 μg/mL) did not alter significantly the median adhesion forces. However, when the coating concentration of fibrinogen increased to 20 μg/mL the median maximal force decreased to 138 pN with the 25^{th} percentile at 93 pN and the 75^{th} percentile at 262 pN, a ~6-fold decline compared to uncoated PET and PET coated with 0.3-0.6 μg/mL fibrinogen (Fig. 36C). The median adhesion force for cell adhesion to uncoated PTFE was smaller (417 pN) than those determined on uncoated PET surfaces (Fig. 36D) and did not change when PTFE was coated with 0.3 μg/mL fibrinogen. However, coating PTFE with 2 mg/mL fibrinogen resulted in a ~5-fold drop in the median adhesion force (84 pN). These direct measurement of adhesion forces between cellular integrins and fibrinogen substrates is in agreement with the data obtained using the AFM tip, thus, corroborating the idea that deposition of fibrinogen on the surface of biomaterials strongly decreases their adhesiveness for cells.

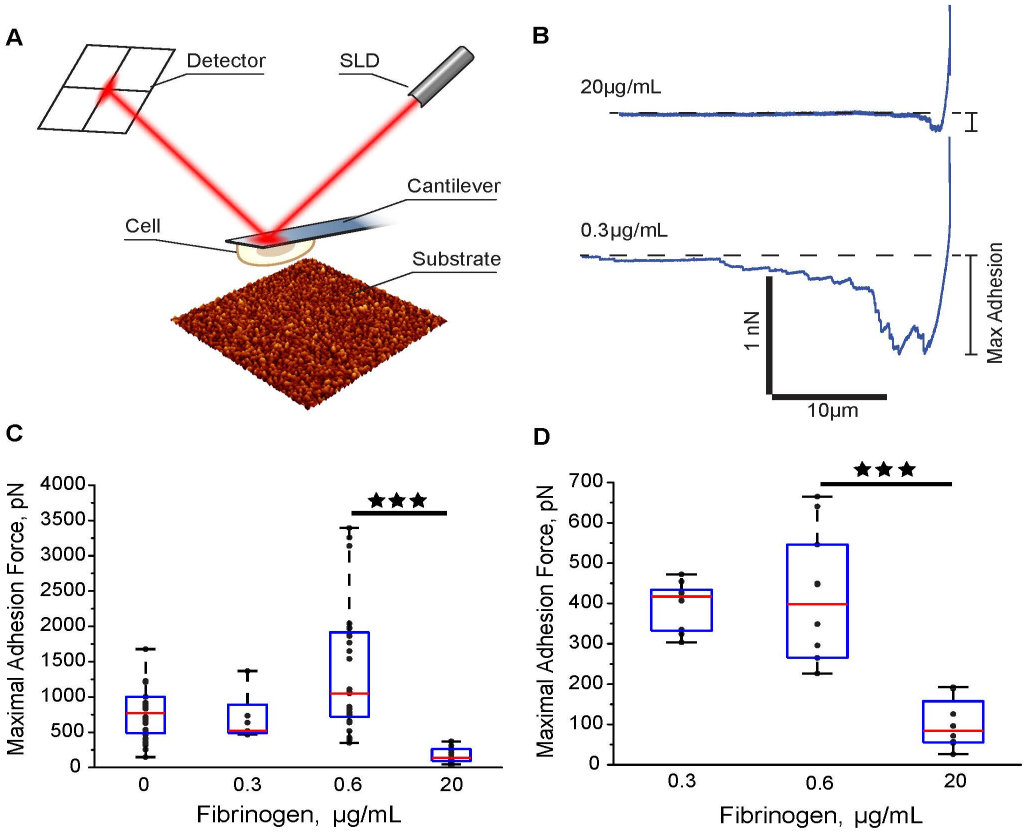


Figure 36. Cell adhesion to fibrinogen substrates formed on the surface of PET and PTFE measured by SCFS. (**A**) Schematic representation of a cell attached to the cantilever during its approach to the fibrinogen matrix. (**B**) Representative force-distance curves for the Mac-1-HEK cells brought into contact with fibrinogen adsorbed on PET at 0.3 and 20 µg/mL. Cells were pressed onto each surface with a force of 1 nN for 120 sec and then withdrawn with a constant speed of 2 mm/s. (**C**) maximal adhesion forces for cell adhesion to untreated PET, and PET coated with 0.3 µg/mL (2 cells, 9 curves), 0.6 µg/mL (8 cells, 30 curves) and 20 µg/mL (3 cells, 22 curves) fibrinogen solutions. (**D**) Maximal adhesion forces for cell adhesion to untreated PTFE, and PTFE coated with 0.3 µg/mL (6 cells, 29 curves) and 20 µg/mL (4 cells, 28 curves) fibrinogen solutions. Adhesion forces are shown as the median (red lines) of each data set, and the top and bottom of the box represent the 75th and 25th percentile. $_{***}$, $p<0.001$.

DISCUSSION

In the this study, we examined how adsorption of fibrinogen on the surface of two materials, PET and PTFE, used for the production of commercial vascular grafts modifies their physical and adhesive properties. The main finding is that fibrinogen adsorbed at low concentrations (≤2 μg/mL) supports platelet and leukocyte adhesion, but when adsorbed at higher concentrations fibrinogen makes the surfaces virtually non-adhesive. Furthermore, cell adhesion drops rapidly over a very narrow range of fibrinogen coating concentrations. These data are in agreement with previous observations obtained on other surfaces, including mica, glass, plastic and fibrin clots (V. K. Lishko et al., 2007; I. S. Yermolenko et al., 2012). Furthermore, examination of adhesive properties of fibrinogen-coated PET and PTFE using AFM force spectroscopy and SCFS is consistent with the data collected on other surfaces, i.e., adsorption of fibrinogen at increasing concentrations reduces the adhesion forces developed between the unmodified AFM tip or a cell and fibrinogen substrates. These findings indicate that adsorption of fibrinogen on biomaterials modify their physical and adhesive properties in a way similar to that observed on other surfaces, underscoring the generality of the fibrinogen-dependent anti-adhesive mechanism.

Previous studies revealed the molecular basis for the differential cellular responses to low- and high-density fibrinogen adsorbed on various surfaces. In particular, we showed that adsorption of fibrinogen at low concentrations results in the formation of a molecular monolayer in which single molecules are directly attached to the surface (I. S. Yermolenko et al., 2010). As the fibrinogen concentration increases, the molecules self-assemble through the interaction between their αC domains (I. S. Yermolenko et al.,

2012), which results in the formation of a multilayer matrix with a thickness of ~10 nm consisting of 7-8 molecular layers packed one on top of the other. The monolayer and multilayer exhibit different adhesion forces when probed by AFM-based force spectroscopy and SCFS. The monolayer is highly adhesive for cells and develops high adhesion forces with the AFM tip. The greater cell adhesion to the monolayer arises as response to the resistance derived from the hard surfaces to which fibrinogen molecules are attached firmly. Thus, this substrate may not yield when cellular integrins or the AFM tip pull on it. Conversely, the multilayered fibrinogen matrix is characterized by weak adhesion forces and is incapable of supporting firm cell adhesion. The origin of the non-adhesive properties of the multilayer lies in its extensibility resulting from the separation of the molecular layers held by non-covalent interactions between the long flexible αC regions of fibrinogen. The multilayer is incapable of transducing strong mechanical forces via integrin receptors. Hence, when platelet integrins pull on the multilayer matrix it yields to the pulling force resulting in weak intracellular signaling, adhesion and cell spreading. The inability of platelets or leukocytes to firmly adhere and consolidate their grip on the extensible matrix leads to their detachment under flow. In spite of its nanoscale nature, the fibrinogen matrix has potent anti-adhesive properties and is capable of repelling cells, even when only 2-3 layers of fibrinogen molecules have been assembled (I. S. Yermolenko et al., 2012).

One additional mechanism that may contribute to the anti-adhesive properties of the fibrinogen matrix is the weak association between fibrinogen molecules in the superficial layers of the matrix compared with that in the deeper layers. Such reduced stability would allow integrins to pull fibrinogen molecules out of the matrix with forces

comparable to or smaller than required for breaking integrin-fibrinogen bonds. Indeed, using a method based on a combination of total internal reflection fluorescence microscopy and atomic force microscopy-based single-cell force spectroscopy, we have recently shown that HEK293 cells expressing leukocyte integrin Mac-1 have the capability to pull fibrinogen molecules from the fibrinogen matrix (Christenson et al., 2014). However, further studies are needed to determine the capacity of platelets and leukocytes to pull fibrinogen molecules from the matrix.

Analyses of adhesion forces developed between uncoated biomaterials and the AFM tip showed a range of 600-1000 pN. These adhesion forces are similar to those detected on glass but in sharp contrast to uncoated mica which displays very low forces (~25 pN). However, even though the adhesion forces determined on uncoated surfaces differ, adsorption of fibrinogen at low concentrations (monolayer) on all surfaces supports high adhesion forces and then, as the concentration of fibrinogen increases, invariably initiates a sharp decline of adhesion forces. At > 2 μg/mL fibrinogen, all surfaces, including those of biomaterials (Fig. 31), are characterized by very low adhesion forces. Thus, regardless of the type of the material, fibrinogen modifies surfaces rendering them non-adhesive. A comparable pattern was detected when adhesion forces were measured by SCFS using integrin Mac-1-expressing HEK293 cells (Fig. 36). In particular, SCFS showed high adhesion forces for uncoated biomaterials (~770 pN and 420 pN for PET and PTFE, respectively), which decreased by 6- and 5-fold respectively, when the coating concentration of fibrinogen increased. As in the force spectroscopy experiments, the difference in adhesion forces observed in SCFS appears to be due to differences in the chemical bond composition of the PET and PTFE surfaces

(Anamelechi, Truskey, & Reichert, 2005). At variance with biomaterials, adhesion forces probed by SCFS on uncoated mica were low (~100 pN) and adsorption of low-density fibrinogen initially increased them to ~1.7 nN. Nonetheless, adhesion forces dropped to ~200 pN when mica was coated with ≥ 2 μg/mL fibrinogen (I. S. Yermolenko et al., 2010). The discussion described in the text above emphasizes the diversity of adhesion forces detected on uncoated biomaterials and after modification by low concentrations of fibrinogen. These data may reflect the materials' surface chemistry and their ability to induce unfolding of fibrinogen, since deposition of high concentrations of fibrinogen consistently creates a non-adhesive surface.

The lack of correlation between the amount of adsorbed fibrinogen and platelet adhesion has been previously reported for a broad range of surface chemistries (Sivaraman & Latour, 2010) including for fibrinogen adsorbed from plasma on hydrophobic surfaces (W. B. Tsai, Grunkemeier, & Horbett, 1999; W. B. Tsai, Grunkemeier, & Horbett, 2003; Wu, Simonovsky, Ratner, & Horbett, 2005). The interpretation of this finding has traditionally been linked to the alterations of fibrinogen conformation and/or availability of integrin-binding sites (Lindon, McManama, Kushner, Merrill, & Salzman, 1986; Wu, Simonovsky, Ratner, & Horbett, 2005; Sivaraman & Latour, 2010). However, even though fibrinogen is known to unfold on various surfaces exposing cryptic binding sites for platelets and leukocytes (Ugarova et al., 1993; V. K. Lishko, Kudryk, Yakubenko, Yee, & Ugarova, 2002), the accessibility of these sequences for platelet integrin $\alpha_{IIb}\beta_3$ and leukocyte integrin Mac-1 does not depend on the coating concentration of fibrinogen (Moskowitz, Kudryk, & Coller, 1998). On the contrary, the contact of fibrinogen with various surfaces induces unfolding of the αC regions, which

then participate in the formation of the extensible multilayer (I. S. Yermolenko et al., 2012). The formation of this non-adhesive multilayer discovered until only recently, is a primary mechanism regulating mechanosensing by blood cells of and their adhesion to fibrinogen-coated surfaces.

Based on the unique pattern of platelet and leukocyte adhesion to fibrinogen-coated surfaces, it is reasonable to suggest that fibrinogen deposition on the surface of vascular grafts should differently impact their adhesive properties immediately after implantation and after graft maturation. Accordingly, as soon as the first molecules of fibrinogen contact and adsorb on the surface, they will apparently create a monolayer, which mediates firm platelet adhesion. As adsorption of fibrinogen continues, the surface of grafts may potentially be coated with the fibrinogen multilayer which is not capable of supporting firm cell adhesion, and from which platelets and leukocytes would be washed away by flow. Therefore, the adhesive capacity of implanted vascular grafts after their initial contact with blood may be strongly affected by the rate of fibrinogen adsorption. It is possible that if fibrinogen is deposited quickly, and consequently the non-adhesive fibrinogen multilayer is formed rapidly, few platelets would be attracted to such surfaces. Furthermore, the surface chemistry that promotes efficient self-assembly of the fibrinogen multilayer may decrease initial thrombogenicity. Speculatively, surfaces pre-coated with the fibrinogen matrix before implantation may be even less adhesive than the naked polymers. In contrast to this idea, which seems counter-intuitive, much of the effort in biomaterials research over the past four decades has been directed toward the development of materials that do not react with platelets and fibrinogen (Jordan & Chaikof, 2007). However, success in reducing platelet adhesion to pristine biomaterials

does not necessarily indicate that platelet adhesion will be reduced after implantation. This is because the initial contact of the fibrinogen molecules with vascular grafts will inevitably create an adhesive fibrinogen monolayer. In this regard, our data show that although uncoated PET is poorly adhesive for platelets and leukocytes, adsorption of very low concentrations of fibrinogen supports strong cell adhesion (Fig. 32). It is only after the fibrinogen multilayer matrix has been formed (>10 μg /mL) that platelet adhesion is strongly reduced.

Although the formation of the non-adhesive multilayer is a natural progression of fibrinogen adsorption, this process is unlikely to be realized in the circulation. Even before the multilayer is formed, the initial formation of the fibrinogen monolayer, which invariably supports rapid platelet adhesion, should lead to their activation, resulting in thrombin generation and fibrin formation. Indeed, numerous studies have demonstrated that very soon after implantation, a thrombotic material consisting of fibrin, platelets and leukocytes is deposited on the inner surface of the prostheses (reviewed in (Zilla, Bezuidenhout, & Human, 2007)). While some variability in the extent and timing of thrombus formation has been noted, the reaction itself does not depend on the type of biomaterial and was observed on both implanted PET and ePTFE grafts. During the first few days after implantation, the thrombus is stabilized and over subsequent weeks to many years, its thickness stays constant. The outermost layer of stabilized thrombi in contact with blood has often been described as loosely packed or compacted fibrin having a smooth, glistening and transparent appearance. The presence of fibrin on the flow surface of vascular grafts has been known for five decades since De Bakey et al described the fibrin lining on Dacron grafts recovered from patients following implant periods of up

to 7 years (De Bakey, Jordan, Abbott, Halpert, & O'Neal, 1964). Numerous studies subsequently confirmed this finding (De Bakey, Jordan, Abbott, Halpert, & O'Neal, 1964; Szilagyi, Smith, Elliott, & Allen, 1965; Berger, Sauvage, Rao, & Wood, 1972; Shi et al., 1997; Stewart, Essa, Chang, & Reichle, 1975; Guidoin et al., 1983). Furthermore, it has been repeatedly emphasized that the fibrin matrix, especially in the middle regions of grafts, lacks all forms of cellular coverage (Zilla, Bezuidenhout, & Human, 2007). Discussion of the acellular appearance of the inner fibrin capsule of grafts implanted in humans has traditionally focused on the conspicuous lack of endothelial cells, which signifies the inability to achieve complete healing. Perhaps even a more prominent feature of mature grafts is the almost complete absence of adherent platelets or leukocytes, i.e., the non-thrombogenic nature of the fibrin lining. The lack of endothelial cells and platelets on the surface of fibrin lining may have the same underlying mechanisms; however, the origin of this phenomenon has not been addressed. One reason for the lack of interest in this problem has been the good overall performance of large-diameter prostheses. In this regard, some studies concluded that the ultimate development of the endothelial lining, a feature of complete healing, is nonessential since vascular grafts remained patent and functional for many years (Stewart, Essa, Chang, & Reichle, 1975; Pasquinelli et al., 1990).

We and other groups have shown that pure fibrin, which is a highly adhesive substrate for platelets and leukocytes, loses its capacity to support cell adhesion in the presence of soluble fibrinogen or after coating of fibrin clots with fibrinogen (V. K. Lishko et al., 2007; V. K. Lishko et al., 2012). The mechanism by which fibrinogen neutralizes the adhesion-promoting capacity of fibrin is evidently the deposition of a non-

adhesive fibrinogen multilayer on its surface. Thrombus formation on the surface of vascular grafts immediately after implantation seems to closely recapitulate the events occurring on damaged areas of natural blood vessels: i.e., initial platelet adhesion followed by their activation and platelet aggregation. The formation of the platelet plug is spatially coordinated with the activation of the blood coagulation system leading to thrombin generation and the formation of fibrin, which is deposited over the platelet plug. Similar to the fibrin lining of vascular grafts, many studies of experimental thrombosis have found no platelets on the surface of thrombus-associated fibrin (van Ryn et al., 2003; Kinlough-Rathbone et al., 1983). Since thrombi in the circulation are continuously exposed to high (2-3 mg/mL) concentrations of fibrinogen, a non-adhesive fibrinogen matrix appears to assemble on the surface of fibrin developed around stabilized thrombi, thereby preventing platelet adhesion. Indeed, our recent examination of the spatial distribution of fibrin and fibrinogen on the surface of stabilized thrombi generated in flowing blood shows the presence of intact fibrinogen on the surface of the fibrin cap. These data imply that the formation of an anti-adhesive fibrinogen layer on top of fibrin prevents continued thrombus growth and, thus, represents a final step of hemostasis. These findings also suggest that the lining on the surface of mature vascular grafts may, in fact, not be pure fibrin, but a fibrinogen layer which acts as a natural anti-adhesive coat. This would explain the lack of platelets and other cells on the surface of mature vascular grafts. Thus, similar to normal hemostasis, implantation of vascular grafts appears to evoke the normal protective reaction of host to injury. However, in contrast to hemostatic thrombi that occasionally form on the surface of locally damaged endothelium in the circulation and eventually heal, prosthetic vascular grafts are “frozen” in the state

of persistent healing for years. At present, the mechanisms that maintain the non-adhesive state of fibrin lining on the surface of vascular grafts for extended periods of time remain to be elucidated.

In conclusion, this study shows that adsorption of fibrinogen on the surface of biomaterials PET and PTFE, the synthetic materials used for manufacturing of contemporary vascular grafts, modifies their adhesive properties. In particular, while fibrinogen deposited at very low concentrations creates a substrate highly adhesive for platelets and leukocytes, adsorption of fibrinogen at increasing concentrations results in the formation of an anti-adhesive matrix. The anti-adhesive properties of the high-density fibrinogen matrix are derived from its multilayer organization and extensibility, which, in turn, results in the inability to transduce strong mechanical forces through integrins, a requirement for firm cell adhesion. Our findings concerning the adsorption of fibrinogen on biomaterials supports the generality of the fibrinogen-dependent anti-adhesive mechanism. The formation of the anti-adhesive fibrinogen multilayer, a structure still poorly appreciated in the biomaterials field, may explain previous observations of a poor correlation between the amount of adsorbed fibrinogen and platelet adhesion (W. B. Tsai, Grunkemeier, & Horbett, 2003; Wu, Simonovsky, Ratner, & Horbett, 2005; Sivaraman & Latour, 2010). Further elucidation of the underlying molecular details of this process should assist in better understanding of hemocompatibility of existing and new biomaterials.

CHAPTER 6

CONCLUSION AND FUTURE RESEARCH

CONCLUSION

The studies presented in this dissertation examine the complex and dynamic process of hemostasis with focus on the cellular and molecular mechanisms regulating thrombus growth and stability. In particular, our studies provide novel insights into the mechanisms that modulate the adhesive properties of stabilized hemostatic thrombi, which are essential to achieving optimal balance between thrombus growth and stability. Moreover, these studies further extend our understanding of the significant physiological role of platelets in thrombus formation.

Formation of a hemostatic thrombus is a highly regulated process that depends on the intricate and dynamic interplay of multiple cellular and molecular elements. This ensures the formation of a thrombus that is stable enough to seal the breach and prevent loss of blood, but is not overly robust to disturb local hemodynamics. The hemostatic response is initiated by formation of a platelet plug, which serves as a scaffold for the activation of the blood coagulation system leading to thrombin generation and fibrin formation. The fibrin network mechanically stabilizes the entire structure of the plug. Although many of the cellular and molecular mechanisms underlying thrombus formation have been successfully identified and extensively studied, there remains a void in understanding how growth of a hemostatic thrombus is terminated beyond that needed for stabilization. In this dissertation I discuss the findings and observations supporting the existence of natural anti-adhesive mechanisms and their physiological relevance in

surface-mediated control of thrombus growth and stability, which may represent a new aspect of hemostasis.

One possibility for regulating thrombus growth and stability is by way of controlling cell adhesion to the thrombus surface. Controlling cell adhesion would allow early hemostasis to proceed unchallenged until optimal thrombus growth and stability is established. If it remained unregulated, however, excessive platelet adhesion, accumulation, and subsequent activation at the thrombus surface would result in continuous thrombus growth and potentially vessel occlusion. Likewise, uncontrollable leukocyte adhesion, accompanied by concurrent release of potent fibrinolytic enzymes, could lead to premature thrombus fibrinolysis and destabilization resulting in bleeding. This suggests the existence of natural mechanisms that operate at the surface of hemostatic thrombi and regulate the adhesive properties, and therefore the thrombogenicity, of the deposited fibrin. Taking this into account, our investigations led to the identification of two anti-adhesive processes that limit cell adhesion to the surface of fibrin clots.

The first anti-adhesive process is fibrinogen dependent. The discovery that fibrinogen may play an important anti-adhesive role had begun to emerge after several studies demonstrated that increasing concentrations of fibrinogen reduce platelet and leukocyte adhesion on the flow surface of fibrin (V. K. Lishko, Burke, & Ugarova, 2007; V. K. Lishko, Yermolenko, & Ugarova, 2010). In subsequent studies we found that fibrinogen accumulates in a nanoscale superficial layer of fibrin clots, which makes surfaces anti-adhesive (Lishko, Yermolenko, & Ugarova, 2010; I. S. Yermolenko et al., 2010). The underlying mechanism accounting for the anti-adhesive properties of this

layer arises from the formation of an extensible multilayered matrix consisting of loosely bound fibrinogen molecules (I. S. Yermolenko et al., 2010; I. S. Yermolenko et al., 2012). This extensible matrix does not enable platelets and leukocytes to transduce strong integrin-mediated mechanical forces. Therefore, cells are incapable of firm adhesion and spreading eventually leading to their detachment. Further investigations revealed that the anti-adhesive property of fibrinogen is concentration dependent. We found that at low concentrations ($\leq$ 2 μg/mL), fibrinogen is deposited as a monolayer in which all of the fibrinogen molecules are in direct interaction with the surface. This monolayer is characterized by strong adhesive forces that enable robust cell adhesion. In contrast, fibrinogen deposited at increasing concentrations (> 2 μg/mL) results in the formation of an extensible anti-adhesive multilayer that does not support continued platelet and leukocyte adhesion, thereby serving to limit thrombus growth. The second anti-adhesive process depends on the proteolytic potential of the plasma protein plasminogen. When transiently adherent cells come in contact with plasminogen, which also accumulates at the surface of fibrin clots, they activate it resulting in the production of plasmin. Plasmin then degrades fibrin via a proteolytic mechanism leading to the formation of a mechanically unstable surface that does not support cell adhesion.

Chapter 2 discusses the dynamic interplay between the non-proteolytic fibrinogen-dependent and the proteolytic plasminogen-dependent anti-adhesive processes and further extends their physiological relevance in hemostasis. Since thrombi formed in the circulation are continuously exposed to both plasma fibrinogen and plasminogen, it seemed intuitive to study their combined effect on cell adhesion. The major finding of this study was that when fibrin clots were simultaneously exposed to plasma proteins

fibrinogen and plasminogen, as would occur in the circulation, their anti-adhesive effects were not additive. Unexpectedly, the deposited fibrinogen neutralized the anti-adhesive effect of plasminogen by masking it and prevented its activation by transiently adherent U937 monocytic cells. Moreover, these findings further supported the hypothesis that fibrinogen does indeed accumulate and concentrate at the surface of fibrin clots where it exerts its anti-adhesive effect. Although fibrinogen and plasminogen both can exert potent anti-adhesive effects at the fibrin clot surface, there seems to be a clear dominance of the fibrinogen-dependent system that may be due to fibrin surface heterogeneity. Therefore, further research will be necessary to determine under which conditions the plasminogen-dependent system may play a significant role in surface-mediated control of thrombus growth.

The studies discussed in Chapter 2 were conducted with a very simple system that may not reflect the more complex series of interactions that occur in the circulation. For this reason, in Chapter 3 we extended our studies on the proteolytic plasminogen dependent-anti-adhesive mechanism with emphasis on the role of platelets in regulating thrombus growth, stability, and dissolution. Chapter 3 discusses the findings supporting the idea that two different functions of platelets are relevant to different regions of the thrombus. We found that fibrin-surface adherent platelets promote the plasminogen-dependent anti-adhesive effect via their ability to assemble components of the plasminogen activation system on their surface and activate fibrin-bound plasminogen. Additionally, platelets could activate plasminogen through the interfacial mechanism, i.e. when components of the plasminogen activation system are assembled on different surfaces. Subsequently, the plasmin-mediated fibrin degradation results in fragmentation

and destabilization of the clot surface making it incapable of supporting strong integrin-mediated platelet adhesion and further platelet accumulation. This leads to platelet detachment under flow, therefore limiting thrombus growth and possible vessel occlusion. Moreover, a major finding of this study is that the proteolytic plasminogen dependent anti-adhesive process occurring at the interface between the platelet and fibrin clot surface remains effective even in the presence of potent inhibitors such as PAI-1 and α2-antiplasmin. Although platelets in the interior of the clot release an abundant amount of PAI-1, it's anti-fibrinolytic effect is limited to the interior of the clot where it prevents degradation of the internal platelet-fibrin network that is the basis for the thrombus stability needed to seal a vascular breech. This study provides new insights into the intricate interactions that regulate the dynamic balance between pro-fibrinolytic processes at the surface of the clot and anti-fibrinolytic processes within the interior. In doing so, this study delineates the significant role that platelets play in this balance.

While the potency of the anti-adhesive fibrinogen multilayer suggests its physiological relevance, the majority of studies so far have been performed using only purified proteins and isolated blood cells under static conditions. As for thrombi formed under flow conditions, they have a distinctive composition and architecture. Moreover, despite the fact that fibrin's interaction with intact fibrinogen has long been known (M. Mosesson, 2005; John W Weisel & Rustem I Litvinov, 2013; Blombäck, Carlsson, Fatah, Hessel, & Procyk, 1994), evidence that plasma fibrinogen may be deposited on the surface of fibrin-rich thrombi in the circulation had yet to be addressed. Therefore, studies presented in Chapter 4 examine whether the anti-adhesive fibrinogen layer can be deposited on the surface of real hemostatic thrombi formed under conditions of shear and

flow. As described in Chapter 4, we provide the first direct experimental evidence for the presence of intact fibrinogen on the surface of stabilized hemostatic thrombi and propose that the fibrin cap, in addition to serving as a mechanical scaffold, functions as a platform for the self-assembly of the anti-adhesive fibrinogen matrix.

Given the nanoscale nature of the fibrinogen multilayer, which would make the observation and manipulation of this structure *in vivo* challenging, we utilized a parallel plate flow chamber to generate an *in vitro* model of fibrin-rich thrombi. Using specific monoclonal antibodies capable of discriminating between intact fibrinogen and fibrin, the spatial distribution of fibrinogen and its association with fibrin were analyzed. The surfaces of thrombi were manipulated to display either intact fibrinogen or fibrin and to demonstrate that the fibrinogen layer is anti-adhesive for platelets. Altogether, deposition of intact fibrinogen on the fibrin surface surrounding stabilized hemostatic thrombi renders them non-adhesive for subsequent attachment of platelets. In contrast, if the surface of fibrin is not covered with fibrinogen as, speculatively, may occur in pathological thrombi, platelets adhere with alacrity. This study highlights the significant role of fibrin(ogen) in the surface mediated-control of thrombus growth and may potentially explain why hemostatic thrombi formed in the circulation do not continue to grow. This study may also guide future analyses of spatial distribution of fibrinogen as well as other proteins on the flow surface of *in vivo* model fibrin-rich thrombi.

The studies discussed above highlighted the role of fibrinogen in establishing a protective mechanism on the fibrin surface that ensures optimal thrombus growth thereby avoiding thrombosis. This prompted us to investigate whether fibrinogen can create its anti-adhesive protective mechanism on the luminal surface of implanted vascular grafts,

which are known to be highly thrombogenic. It is widely recognized that after implantation, prosthetic vascular grafts spontaneously acquire a layer of adsorbed plasma proteins, which ultimately result in graft failure. One of the main proteins to become adsorbed is fibrinogen due to its high circulating plasma concentration (2-3 mg/mL). This protein layer can facilitate a wide range of biological responses including cell adhesion, blood coagulation, and potentially thrombosis. Therefore, a comprehensive understanding of blood-biomaterial interactions is necessary for the development of more compatible biomaterials that can be used in making long-term vascular grafts.

The studies of Chapter 5 were conducted to examine deposition of fibrinogen and its anti-adhesive effect on the surface of two biomaterials: PTFE (polytetrafluoroethylene) and PET (polyethylene terephthalate). The adhesive and physical properties of these biomaterials used in the production of contemporary vascular grafts were examined before and after adsorption of fibrinogen. The principal finding is that adsorption of fibrinogen at low concentrations (≤2 μg/ml) results in platelet and leukocyte adhesion, but adsoption at higher concentrations (see Fig.37) fibrinogen makes the surfaces non-adhesive. These findings suggest that the adhesive capacity of implanted vascular grafts after their initial contact with blood may be strongly affected by the rate at which fibrinogen is adsorbed. It is possible that if fibrinogen is deposited and consequently the anti-adhesive multilayer is formed rapidly, few platelets would be attracted to such surfaces. Therefore, a biomaterial surface chemistry that promotes efficient self-assembly of a fibrinogen multilayer might actually decrease initial surface thrombogenicity. Speculatively, biomaterials pre-coated with a fibrinogen matrix before implantation might reduce the incidence of graft thrombogenic failure. The formation of

an anti-adhesive fibrinogen multilayer may explain previous observations concerning the discrepancy between the amount of deposited fibrinogen and adhesion of platelets (W. B. Tsai, Grunkemeier, & Horbett, 2003; Wu, Simonovsky, Ratner, & Horbett, 2005; Sivaraman & Latour, 2010). Further elucidation of the underlying molecular mechanisms of this process may lead to the design of a new generation of hemocompatible biomaterials that could increase the long-term patency of implanted vascular grafts.

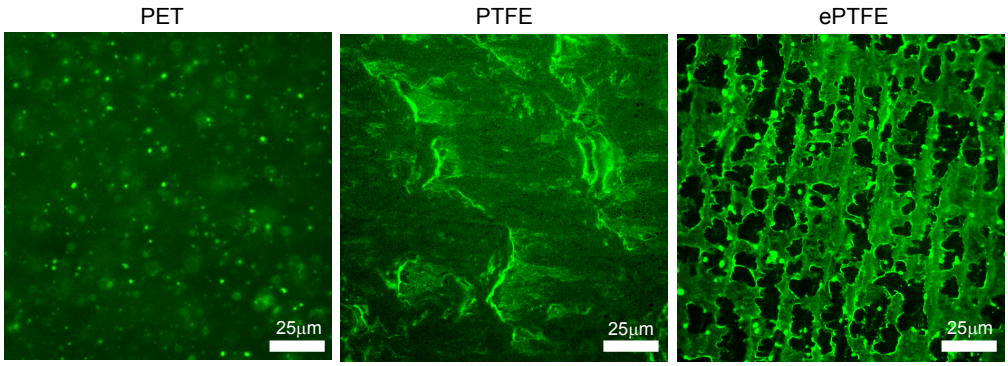


Figure 37. Deposition of fibrinogen onto the surface of biomaterials PET, PTFE, and ePTFE. Biomaterials were coated with 2 mg/mL fibrinogen, washed to remove excess fibrinogen, and then incubated with anti-FPA mAb. Fibeinogen deposition was detected using Alexa 488-cojugated secondary antibody.

The mechanisms that contribute to normal hemostasis are also involved in pathological conditions contributing to cardiovascular disease. Therefore, elucidation of the molecular mechanisms that mediate hemostatic balance is crucial to understanding the dysfunction of these processes as well. Specifically, identifying fibrinogen and plasminogen-dependent anti-adhesive mechanisms and their role in regulating hemostatic thrombus growth and stability will provide new insight into mechanisms that drive abnormal thrombus formation as well as providing clues into the best strategies to prevent thrombosis or bleeding. Future research will greatly expand our

knowledge on these distinct pathways in hemostasis and thrombosis, which we believe will be the key to the development of novel anti-thrombotic therapy with minimal hemorrhagic complications, as well as vascular grafts characterized by durability, higher patency rates, and hemocompatibility.

FUTURE RESEARCH

We have demonstrated that our *in vitro* model fibrin-rich thrombi are covered with a layer of fibrinogen that renders their surface anti-adhesive for platelets. However, the processes that control the adhesive properties of thrombi may involve not only fibrinogen but also other circulating plasma proteins that could modify the fibrinogen binding capacity and thus its anti-adhesive effect. Moreover, whether other plasma proteins that are capable of interacting with fibrin are incorporated at the interface between fibrin and fibrinogen, within or on the surface of the fibrinogen shell is unclear. Furthermore, whether fibrinogen is deposited on the surface of fibrin formed on the surface of *in vivo* hemostatic thrombi remains unknown. Therefore, additional studies will be needed to identify and characterize the composition of the anti-adhesive fibrinogen multilayer on the surface of real hemostatic thrombi generated *in vivo* using murine models of thrombosis, and to elucidate the role of other plasma proteins in regulation of thrombus growth and stability.

The majority of our studies have been dependent on standard cell adhesion assays as a method for studying the fibrinogen's anti-adhesive effect. Although adhesion assays are easy to perform and allow for semi-quantitative measurements of integrin-dependent cell adhesion to various immobilized substrates, they inherently preclude quantification

of adhesion forces from a physical standpoint. Therefore, to definitively elucidate the anti-adhesive effect of fibrinogen, we needed to obtain more precise quantitative measurements of the adhesion forces. This is possible by employing atomic force microscopy (AFM)-based techniques. AFM is a multifunctional tool that allows for high resolution and multifaceted imaging as it can be combined with a variety of optical microscopy techniques such as differential interference contrast (DIC), confocal fluorescence microscopy, total internal reflection fluorescence (TIRF), and two-photon microscopy.

AFM also allows for force spectroscopy measurements with piconewton (pN) resolution. Specifically, single cell force spectroscopy (SCFS) permits manipulation of a single living cell and quantification of the interaction forces generated between the cell and a substrate or another cell. For these reasons, we have initiated a study using AFM and AFM-based force spectroscopy to measure adhesion forces on fibrin and fibrinogen-coated fibrin surfaces. We are able to analyze the adhesive/non-adhesive properties of fibrin and fibrinogen-coated fibrin clots using force spectroscopy with a polystyrene bead AFM tip. Indeed, SCFS measurements can be performed using platelets, U937 monocytic cells, or HEK293 cells stably expressing the Mac-1 integrin. In this assay, a single cell is attached to a tipless cantilever of an AFM and maximum adhesion force curves for fibrin and fibrinogen-coated fibrin surfaces are acquired (Fig. 38). Adsorption of fibrinogen on fibrin clot surfaces results in a significant decrease in the adhesion force measurements (Fig 39).

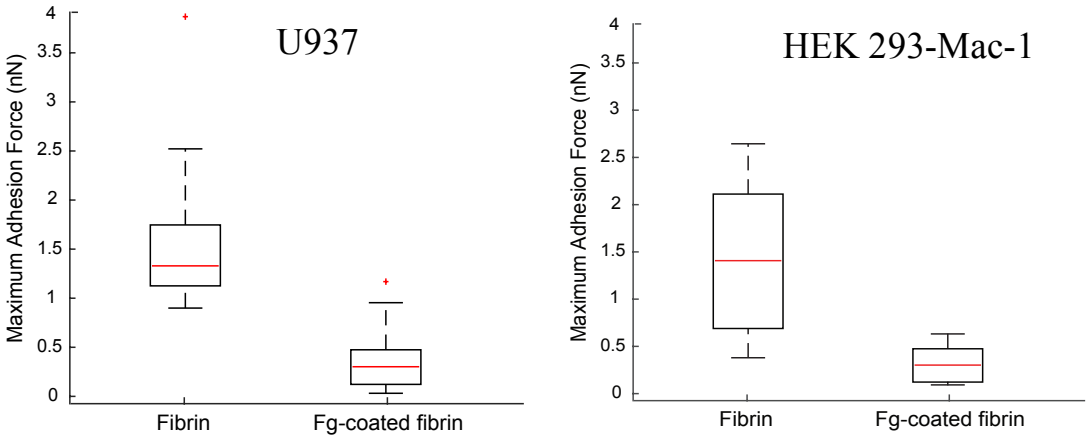


*Figure 38. SCFS measurements for U937 and HEK293-Mac-1 cells on fibrin and fibrinogen-coated fibrin gels***.** A single U937 cell or HEK-Mac-1 cell was attached on a concanavalin-A functionalized tipless AFM cantilever, pressed onto the surface of fibrin or fibrinogen-coated fibrin gel, and allowed to establish firm integrin-mediated adhesion. After a contact time of 3 min, the cell is retracted from the surface and force-distance curves are recorded. These curves correspond to cell adhesion signatures. Final data are presented as bar and whiskers plots which show the maximum adhesion forces (nN) generated between cellular integrins and the surface of fibrin or fibrinogen-coated fibrin.

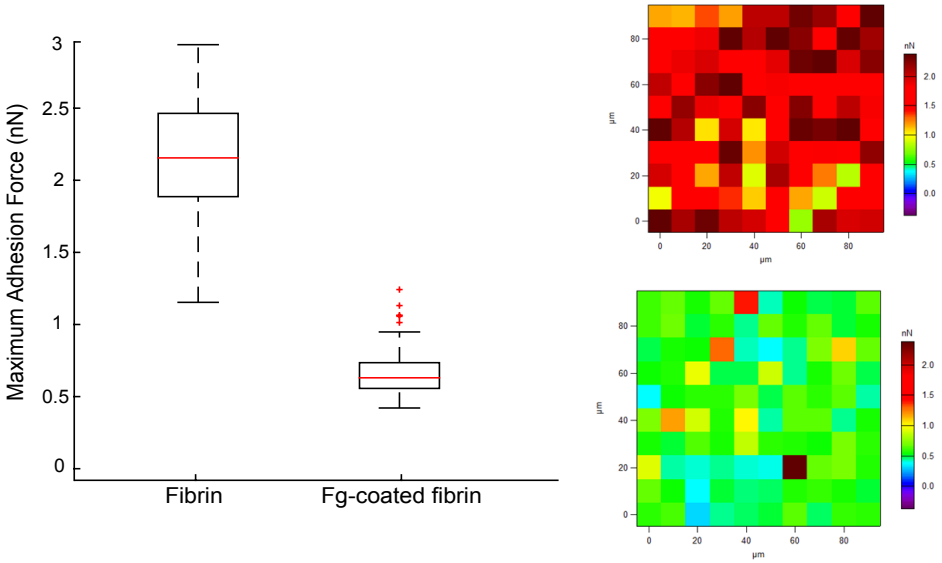


Figure 39. Maximum adhesion force measurements and AFM adhesion maps of fibrin and fibrinogen-coated fibrin gels. The bar and whiskers plot shows the difference in maximum adhesion forces (nN) generated on fibrin versus fibrinogen-coated fibrin gels. The corresponding AFM adhesion force maps are shown on the right. Each adhesion map was generated by scanning an AFM-bead tip (5µm diameter) over a 90µm x 90µm area. Adhesion maps are color-coded to reflect the amount of adhesion force between the AFM-bead tip and the fibrin or fibrinogen-coated fibrin gels. Highest adhesion is shown as dark red whereas lowest adhesion is shown as purple.

The data so far suggest that deposition of fibrinogen creates a poorly adhesive surface, in that the adhesion forces measured between the fibrin surface and the cell surface (via integrins) are relatively low. These preliminary results support and extend the concept that deposition of a fibrinogen matrix on a fibrin clot prevents stable cell adhesion by modifying the physical properties of fibrin surfaces so as reduce adhesive force generation. These studies pave the way for future AFM investigations that elucidate both the structure of the fibrinogen multilayers formed as well as providing us with better understanding of the mechanical properties of the multilayered fibrinogen coatings that act as a crucial functional interface between thrombi and their cellular partners during thrombus growth either in situ or on the surfaces of the biomaterials used in vascular grafts.

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